Iron binding compounds produced by novel actinomycetes

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Declaration

I declare that while registered as a candidate for the degree for the Doctor of Philosophy, no part of this thesis has been submitted in support of an application for any degree or qualification of Liverpool John Moores University or any other University or Professional Institution.

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Finally I would like to thank my husband for sharing his life with me...

Abstract

Streptomyces have developed an assortment of biochemical mechanisms to enable themselves to grow and survive in oligotrophic environments. Free iron is a particularly scarce entity in nature and these bacteria capture iron using an array of siderophores. Siderophores possess important pharmaceutical activity and have potential environmental applications in bioleaching. In an attempt to identify novel siderophore compounds we have isolated streptomycetes from soil samples (Thailand and the UK). Siderophore production was screened and twenty Thai and five UK strains were confirmed positive exhibiting prolific siderophore production. To establish the novel nature of certain strains (23F, 29C, 31B, 31C and 33D) a variety of characteristics were determined to produce a detailed phenotypic and metabolic profile. Analysis of the nucleotide sequences of 16S rRNA was performed and we report on the taxonomic relationships of these isolates.

Isolate 23F, which is phylogenetically related to *Streptomyces coelicolor*, was categorised as a multiple siderophore producer. In rich MS media the strain synthesised a catecholic compound, which formed strong ferric complexes, yet to be characterised. In starch-casein basal media the isolate produced desferrioxamine E (hydroxamate), which is a valuable clinical compound.

There is significant evidence to believe that by feeding supplements (amino acids and Dip) to the fermentation we can increase the yield of the desferrioxamine E, directing the bioprocess toward the desired product. It appears that the combination of glutamic, aspartic acids and serine also has a positive effect on the strain's viability providing a perfect model for a robust fermentation system.

Furthermore it was noticed that the provision of certain supplements did influence the strain's physiology without affecting the siderophore production. The latter implies that morphological differentiation does not appear to be associated with the chelate agent's synthesis.

A very important finding of this study was the production of desferrioxamine E in rich media (MS), where only the catecholic compound is known to be naturally expressed. In the presence of lysine catechol production appeared to be almost unaffected, however the organism synthesised the second siderophore as well.

Strain 23F is believed to produce scavenging moieties responsible for the acquisition of Mn II, Co II, Cd II, Ni III, Al III, Li I, Cu II, Zn II and Mg II. There is some evidence suggesting that siderophore biosynthesis is responsible for this phenomenon.

Some interesting data reveal that in starch casein media copper sequestering by the chelating agent is not regulated by iron. This is of particular commercial value as siderophore- mediated copper decontamination can be achieved independent of the presence of iron.

Moreover the organism's tolerance of different ions in terms of growth and siderophore release has also been investigated. These data encouraged the design of a model bioreactor, which has successfully demonstrated the value of 23F in terms of biodegradation.

In an attempt to investigate the molecular control of the siderophore (desferrioxamine E) genes in strain 23F *desA* and *desE* were sequenced. These data could facilitate the identification of the specific role of each gene in the chelate agent's biosynthetic pathway. This will further enable the monitoring of the gene expression at the level of messenger RNA (RT-PCR).

Abbreviations:

1. CAS: Chromo Azuerol Sulphate.
2. 2,3-DHBA: di-hydroxybenzoyl.
3. DesE: Desferrioxamine E.
4. DFO: Desferrioxamine.
5. DH_2O: distilled water.
6. Dip: 2,2'-dipyridyl.
7. DmdR: Divalent Metal-Dependent Regulator.
8. DNA: Deoxyribonucleic Acid.
9. Fur: Ferric Uptake Regulator.
10. H_2O: water.
11. HIF: Healthy Intercellular Fluids.
12. HPI: High Pathogenisity Island.
13. IIF: Infected Intercellular Fluids.
14. MHF: Muller-Hinton-Fructose.
15. MHG: Muller-Hinton-Glucose.
16. MH: Muller-Hinton.
17. MS: Manitol-Soya bean flower medium.
18. NRPS: Non-Ribosomal Peptide Synthases.
19. LD_{50}: 50% lethal dose.
20. LG: Light Growth.
21. ND: Not Determined.
22. OD: Optical Density.
23. PCR: Polymerase Chain Reaction.
24. PS: Pumice Stone.
25. RA: Rhodotorulic acid.
26. rpm: Revolutions Per Minute.
27. rRNA: Ribosomal Ribonucleic Acid.
28. RT-PCR: Reverse Transcript Polymerase Chain Reaction.
29. SEM: Scanning Electron Microscopy.
30. Tf: transferrin.
31. TLC: Thin Layer Chromatography.
32. WHO: World Health Organisation.

33. YEME: yeast extract-malt extract.

34. Zur: Zinc Uptake Regulator

35. ^{0}C: Celsius.

36. µl: Microlitre.

37. ml: Millilitre.

38. µM: Micromolar.

39. mM: Miillimolar.

40. mm: Millimeter.

41. µg: Microgram.

42. g: Gram.

43. L: Litre

44. H: Hour.

45. cm: Centimeter

List of Figures:

Figure 18: A typical example of a CAS assay. It is worth noticing that both catechol (yellow halo) and hydroxamate (orange halo) producers were identified.

Figure 19: A successful Arnow's assay (for catechol identification). Samples 5 and 10 from Freshfield were screened positive for catechol production (pink colorization). The last two samples are non-producers (controls).

Figure 20: An inconclusive Atkin's assay of Thai isolates 31B, 31C and 33D due to the presence of yellow/brown pigment in the media.

Figure 21: Phylogenetic relationships of the isolates and related Actinomycetes based on the 16S rRNA sequence analysis. The tree is bootstrapped by 1000times by Neighbour-Joining. (N-J).

Figure 22: Light (left) and scanning electron microscopy image (right) of 29C. In both cases cultivation was on starch-casein agar.

Figure 23: An Arnow's (catechol production) positive reaction (left) of 29C growing on starch-casein liquid media confirmed by CAS assay as well (growth on starch-casein agar). It is worth noticing the catechol overproduction on CAS agar.

Figure 24: A representation of a GP2 MicroPlate™, Biolog. It demonstrates the nutrient requirements of 29C. Purple dye (tetrazolium) illustrates a positive reaction. The first well contains water and is used as a control (see Materials and Methods for description).

Figure 25: Scanning electron microscopy image of 33D (left) and cultivation on YEME agar, where strong pigmentation is observed (right).

Figure 26: 33D growing on MS agar, pH 6 (1), pH 7 (2) and pH 8 (3). The organism prefers pH 8, where it sporulates much quicker.

Figure 27: CAS assay demonstrating the production of a small catecholic compound on MS agar.

Figure 28: The effect of iron on 33D growing on MS agar. 1: 1 μM $FeCl_3$, 2: 2.5 μM $FeCl_3$, 3: 5 μM $FeCl_3$, 4: 10 μM $FeCl_3$, 5: 100 μM $FeCl_3$ and 6: 1 mM $FeCl_3$.

Figure 29: A CAS assay demonstrating the effect of iron on siderophore production of 33D growing on MS agar. 1: 1 μM $FeCl_3$, 2: 2.5 μM $FeCl_3$, 3: 5 μM $FeCl_3$, 4: 10 μM $FeCl_3$, 5: 100 μM $FeCl_3$ and 6: 1 mM $FeCl_3$. The blue halo around six is due to the iron transfer for 33D to the CAS agar.

Figure 30: Growth of 31C on MHG or MHF (left) and on YEME (right) solid media.

Figure 44: Fluorescent images of 13 days old 23F cultures growing in a control flask (no supplements) [A] and in the presence of 5 g/L GAS (B). The differences in viability are significant. Image A was collected by deconvolution microscopy. The green areas of the pellets indicate hyphae with intact functioning membranes; the red/ orange areas are those with compromised membranes.

Figure 45: Illustration of the fermentation vessel employed for the cultivation of 23F in liquid media.

Figure 46: Illustration of siderophore production of 23F during feeding fed batch fermentation. Arnow's and Atkin's assays were used for catechol and hydroxamate production respectively.

Figure 47: A pellet of 23F grown in a fed-batch culture (left) [Magnification 40x] and highly viscous broth harvested at the end of fermentation (right).

Figure 48: CAS assay demonstrating that 23F still produces a catechol when grown in the presence of 100 μM $FeCl_3$.

Figure 49: The affect of Co II on aerial mycelia formation (MS solid media). At 10-5μM 23F grows around the well (left) but does not form aerial mycelia (right, light microscopy image).

Figure 50: Comparison of the stain's growth in the presence of 500 μM (left) and 0.5 μM (right) of Mg II in starch casein media.

Figure 51: 23F culture growing the presence of 1mM Mg II (MS).

Figure 52: In the presence of high levels (500 μM) of iron in rich media (MS) only substrate mycelia were observed around the well.

Figure 53: Description of the physiological effect of 0.5 μM of Cu II on 23F. As you move away from the well there is a zone of cells producing aerial mycelia. Further out from the centre aerial mycelia disappear and only a zone of substrate mycelia is evident. Moving further of the centre a zone of aerial mycelia is again seen.

Figure 54: Description of the effect of 1mM- 10μM Cu II on the cells in rich media.

Figure 55: Schematic representation of desferrioxamine biosynthesis gene cluster of *S. coelicolor* A3(2).

Figure 56: The nucleotide sequence of *DesA* from isolate 23F.

Figure 57: The nucleotide sequence of *DesD* from isolate 23F.

List of Tables:

Table 17: CAS assay results describing the siderophores ability to bind a variety of heavy metals. It is worth noticing that catechol (MS) exhibited high affinity for copper. The diameter of the well was 1cm.

Table 18: $FeCl_3$ and modified CAS assays of 23F growing in the present of 500-0.1μM of various metals. – and + indicate the absence and presence (minor) of a halo respectively. ~ indicates light growth or production. Where numbers were used the production was significant. Well diameter 1cm.

Table 19: $FeCl_3$ and modified CAS assays of 23F growing in the present of iron and various other metals. – and + indicate the absence and presence (minor) of a halo respectively. ~ indicates light growth or production. Where numbers were used the production was significant. Well diameter 1cm.

Table 20: $FeCl_3$ and modified CAS assays of 23F growing in the present of various metals. – and + indicate the absence and presence (minor) of a halo respectively. ~ indicates light growth or production. Where numbers were used the production was significant. Well diameter 1cm.

Table 21: Description of the effect of different of different ion concentrations on the isolate's physiology. + and - indicate growth and the lack of it respectively during a three day cultivation. ↑ indicates improved growth.

Table 22: Description of the primers designed to target *desA* and *desD* gene sequences of the desferrioxamine biosynthetic gene cluster.

Table 23: A very brief elemental composition, given as weight (Wt %), of various pigmented pumice stones (for more information please refer to Appendix III). PS1: brick red pigments, PS2: Brown pigment, PS3: yellow pigment, PS4 green pigment, PS5: Typical grey pigment, PS6: reddish/ orange pigment. (-) Signifies element not present.

Table 24: Elemental analysis showing the data generated for PS1, which contained the brick red pigment.

Table 25: Elemental analysis showing the data generated for PS2, which contained a brown coloured stone found within the pumice.

Table 26: Elemental analysis showing the data generated for PS3, which contained the yellow pigment.

Table 27: Elemental analysis showing the data generated for PS4, which contained the green pigment.

Table 28: Elemental analysis showing the data generated for PS5, which was the typical grey pumice.

Table 29: Elemental analysis showing the data generated for PS6, which contained the reddish-orange pigment.

Table 30: Elemental analysis showing the data generated for the coal sample.

Table 31: Elemental analysis showing the data generated for SP1, which was green coloured sponge.

Table 32: Elemental analysis showing the data generated for SP2, which was aqua coloured kitchen sponge.

Glossary:

1. Aerobactin: A hydroxamate produced by *Aerobacter aerogenes*.
2. Arnow's assay: A test screening for catecholic siderophores.
3. Atkin's assay: A test screening for hydroxamate production.
4. Bioremediation: A process that employes microorganisms or their compounds for enviromental decontamination.
5. Biologs TM: a microplate determining the 'metabolic fingerprinting' of an isolate.
6. Bisucaberin: a hydroxamate produced by *Vibrio salmonicida*.
7. Brucebactin: A catecholic siderophore produced by *Brucella abortus*.
8. CAS assay: A general test screening for siderophore production.
9. Catechols: siderophores that bind iron by using the hydroxyl groups of catechol rings, which normally consist of di-hydroxybenzoyl (2,3-DHBA) conjugates.
10. Chrysobactin:A catechol produced by *Erwinia chrysanthemi* 3937 (plant pathogen)
11. Coelichelin: A tris-hydroxamate produced by *Streptomyces coelicolor*.
12. *DesA*: The iron-regulated promoter involved in desferrioxamine B biosynthesis.
13. *DesD*: The iron-regulated promoter involved in desferrioxamine E biosynthesis.
14. Desferrioxamines: A_1, A_2, B, C, D_1, D_2, E, F and G represent the category of hydroxamates.
15. Enterobactin: a catecholic siderophore also called enterochelin.
16. Enterochelin: a catecholic siderophore also called enterobactin.
17. Ferrichrome: A hydroxamate mainly produced by *Aspergillus*, *Ustilago* and *Penicillium* species.
18. Ferrioxamines: Iron-bound desferrioxamines.
19. Ferritin: The main intracellular iron storage protein in both prokaryotic and eukaryotic cells.
20. Hetorobactin (A and B): a catechol (DHBA conjugate) produced by *Rhodococcus erythropolis* IGTS8.

21. Hydroxamate: siderophores that use nitrogen atoms of thiazoline rings to chelate ferric ion
22. Inducer: a molecule that starts gene expression.
23. Lactoferrin: a globular multifunctional protein with antimicrobial activity, which is due partly to its high affinity for iron.
24. Oxidative stress: the intracellular accumulation of abnormally high levels of oxygen radicals.
25. Pumice stones: they are produced by volcanic eruptions and are characterized as porous lava.
26. Repressor: A DNA protein that regulated gene (s) expression by decreasing the transcription rate.
27. Siderophores: iron carriers (from the Greek word), are low molecular weight compounds (600-1500 Daltons) that chelate the ferric complexes and other ions with a high affinity (Neilands, 1995).
28. Virulence: the degree of pathogenisity.
29. Xenosiderophores: siderophores that can be recognised, 'pirated' and transported by non- producers.

Contents: **Page No.**

Chapter 1

Introduction

1. 1. 0. General Characteristics of Streptomycetes

Streptomycetes are a group of high G+C ratio Gram-positive bacteria. They are aerobic, mycelia-forming organisms that undergo complex morphological differentiation, which includes the sequential production of substrate mycelium, aerial mycelium, and spores. Differences in shape and arrangement of aerial filaments and spore-bearing structures of various species are among the fundamental features used in identifying and classifying members of the genus streptomycetes. Hyphal filaments are usually 0.5-10 μm in diameter and of indefinite length (Hopwood, 1999).

The taxonomy of streptomycetes has been extensively studied by many groups and comprehensive reviews are available in the literature (Anderson & Wellington, 2001, Williams *et al.*, 1983a and Williams *et al.*, 1983b). Modern molecular taxonomical approaches involve the sequence analysis of the 16S rRNA gene, a particularly powerful tool in the classification of streptomycetes. Although 16S rRNA is a highly conserved area of the genome, it contains enough variations to allow us to investigate relationships at the genus, species and strain levels. Nevertheless it can be misleading due to intragenic variation, which highlights the need of genotypic and phenotypic observations (Anderson & Wellington, 2001 and Christova *et al.*, 1995).

Nutritionally, the streptomycetes are extremely versatile. A wide variety of carbon sources can be utilized, such as sugars, amino acids, alcohols and some aromatic compounds (Hodgson, 2000 and Madigan *et al.* 1997). These bacteria are general saprophytes, which means that they gain nourishment directly from dead or decaying organic matter. They secrete extracellular enzymes and adsorb the soluble breakdown products of the interaction of these enzymes with insoluble polymers, such as protein and starch. Therefore, streptomycetes can grow in carbohydrate replete environments where nitrogen and phosphorous are in poor supply (Hodgson, 2000). Streptomycetes are facultative oligotrophs, which means that they grow in both oligotrophic or copiotrophic conditions. Although streptomycetes have generation times as low as one hour in laboratory rich nutrient conditions, in woodland soil their generation time is estimated to be of the order of 1.7 days (Williams, 1985).

The discovery of genetic conjugation in streptomycetes [*Streptomyces coelicolor* A 3(2)] in the late 1950s led to a very detailed understanding of streptomycete genetics, which is divided into three phases: *in vivo*, *in vitro* and *in silico* genetics. Although, the phases overlap, the *in vivo* genetics occupy 1960's and 1970's; the *in vitro* dominates in 1980's and finally the *in silico* engage in the 1990's (Hopwood, 1999).

Because of the great economic and medical importance of many streptomycetes products, they have attracted significant interest amongst the scientific community. They produce secondary metabolites, including 60 % of the 10-12,000 antibiotics known today. In addition a range of pharmaceutically active compounds and enzymes are also produced by members of the genus (Bentley *et al.*, 2002). These compounds include immunosuppressants, cancer therapy drugs, antineoplastic agents, proteases, enzyme inhibitors, pesticides, restriction endonucleases and glycohydrolases. Glucose isomerase, isolated from a thermophilic streptomycete, is used in the food industry for converting glucose to the sweetener fructose (Hopwood, 1999). *S. coelicolor*, the model streptomycete, produces at least four secondary metabolites: the blue polyketide antibiotic actinorhodin, the red-pigmented undecylprodigiosin, the calcium-dependent antibiotic and methylenomycin; none of which possess pharmaceutical significance (Bibb, 1996).

The synthesis of secondary metabolites is not linked to growth (Demain, 1992), however it may occur in either the growth or the stationary phase of batch culture (Hobbs *et al.*, 1990). Moreover, the particular growth phase in which secondary product formation occurs depends upon the nutritional environment in which the producing organism is grown (Obanye *et al.* 1996).

It is anticipated that the isolation, characterisation and the investigation of actinomycetes might lead to novel secondary metabolites with unique antimicrobial activities of pharmaceutical interest. In order to achieve this, a large number of isolates need to be screened. The search will be more promising if diverse environments are sampled, where adaptation is not only desirable but essential as well (Oskay *et al.*, 2004).

Streptomycetes have evolved complex molecular mechanisms to ensure efficient competition in their natural environment. There are many paradigms of streptomycetes producing two structurally and chemically different secondary metabolites that either act synergistically against a target microorganism or contingently to successfully compete in their environment for nutrients. In the first case metabolites that act synergistically gain greater antimicrobial activity than when they work individually, whereas in the second case the contingent products, although very similar, are recognised and regulated independently by the microorganism (Challis & Hopwood, 2003).

For example *S. clavuligemus*, *S. jumonjinesis* and *S. katsurahamanus* produce clavulanic acid and cephamycin C. The former is a natural inhibitor of β-lactamases

whereas the latter is a β-lactam antibiotic and the combination of both is well known to be effective against β-lactam resistant organisms. Although there are isolates that only produce cephamycin C, there are no known streptomycetes that produce clavulanic acid alone. This suggests that clavulanic acid synthesis evolved in a cephamycin C ancestor in order to 'attack' lactamase-mediated resistant bacteria. Indeed in all the above-mentioned microorganisms the clavulanic acid cluster is directly adjacent to the cephamycin C gene cluster (Challis & Hopwood, 2003). Moreover many members of streptomycetes produce streptogramins, which are pairs (type A & type B) of structurally different antibiotics that synergistically inhibit bacterial protein synthesis (Figure 1). The binding of type A to the ribosome leads to a 40-fold increase in the ribosomal binding affinity of type B. Type A or B alone are bacteriostatic, whereas the combination of both is bacteriocidal. Moreover there are no species that produce only one type of the antibiotic. Genome-level analysis has revealed that an ancestor streptomycete, producing a single antibiotic, acquired the ability to synthesize a second antibiotic in order to overcome competition with resistant bacteria inhabiting the same environment (Challis & Hopwood, 2003).

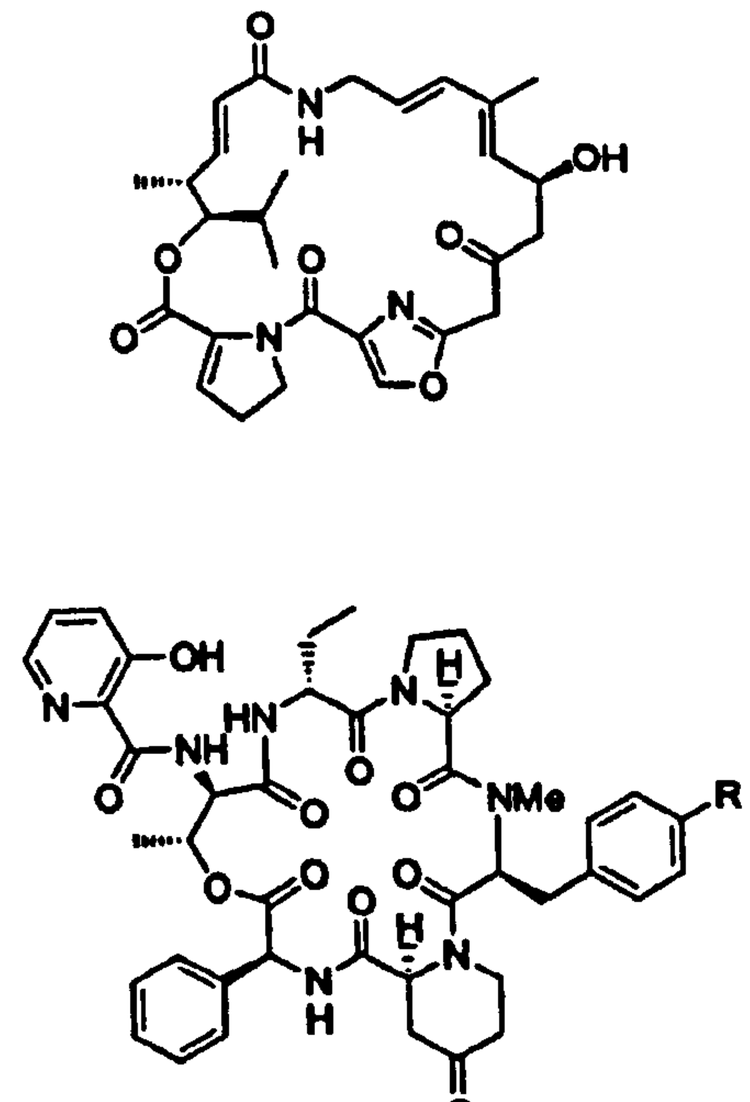


Figure 1: Structures of Type I (top) and Type II (bottom) of streptogramins. Redrawn from Challis & Hopwood, 2003.

1. 1. 1. Growth cycle of *Streptomyces*:

One of the reasons for studying the genetics of this genus is that they exhibit a complex life cycle; an insight into the control of the process will provide fundamental information for developmental biologists. A spore will germinate and outgrowth leads to the formation of a germ tube. Its cell wall grows at the tip at a rate that doubles with each replication of the chromosomes in the initially aseptate germ tube, which continues to lengthen to form primary substrate hyphae. The hyphae grow into the food source to form a branched, compact substrate mycelium (Hodgson, 2000). Hyphae begin to grow vertically upward out of the centre of the substrate mycelium, which functions as nutrient source for the formation of aerial hyphae. The latter gives the colony a hairy appearance (Chater, 1993) [Figure 2]. Physiological changes may be triggered by nutrient limitation or other stresses and manifest as one or all of the following:

- Increased production of some extracellular proteins.
- Onset of secondary metabolism.
- Initiation of lysis of some compartments of the substrate mycelium.
- Onset of storage metabolism in substrate hyphae on the colony surface.
- Initiation of aerial hyphae growth.

Mutant colonies that have lost the 'hairy' appearance of the wild type and appear flat and shiny are referred to as bald *(bld)* mutants. Moreover, white *(whi)* mutants of *'Streptomyces coelicolor* A3 (2) produce an aerial mycelium but it is not the characteristic grey/brown colour of the wild type (Kieser *et al.*, 2000). However three classes *bldA*, *bldC* and *bldD* could be induced to sporulate on minimal media in which glucose has been replaced by an alternative carbon source, such as mannitol (Chater, 1993 and Mohan *et al.*, 1991). Again this highlights the complexity of physiological function regulated at the molecular level

The initially straight aerial hyphae begin to twist to form tight coils and then a very regular synchronized cell division of the hyphae occurs. The result of this cross wall synthesis is the formation of a compartment that has a single copy of the genome. These compartments lay down specific cell wall layers, including a hydrophobic cell sheath, and round up to form ellipsoidal spores. A water droplet on the surface of the colony will then disperse the mature spores (Hodgson, 2000).

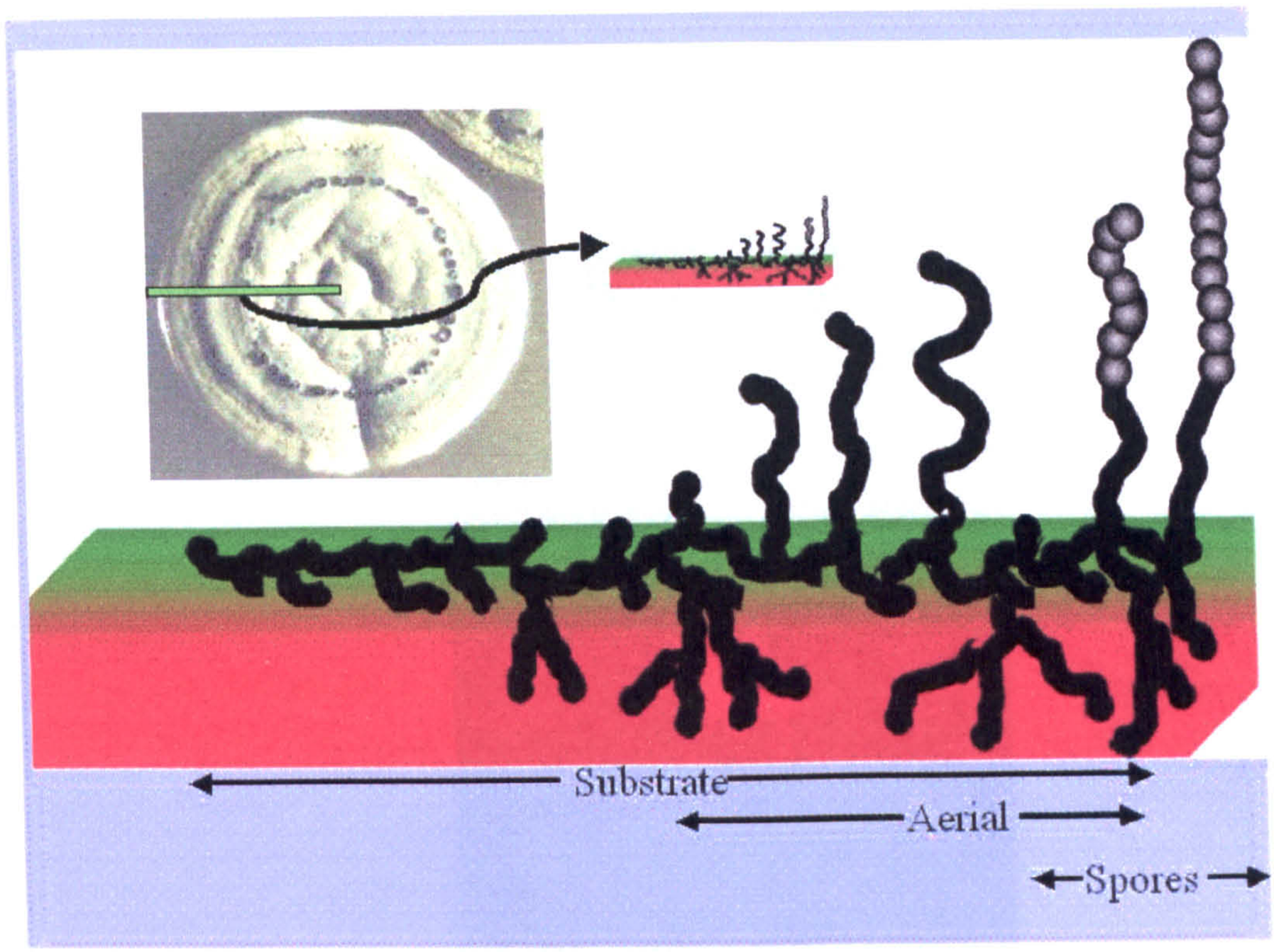


Figure 2: Description of the *Streptomyces*' growth cycle. Section through a Streptomycete colony depicting the various stages of the growth cycle illustrated as a cartoon.

1. 1. 2. Growth in liquid media

Most streptomycetes do not differentiate in liquid culture and only produce vegetative hyphae, growing as compact masses or pellets of mycelium. These pellets are spherical in shape and suffer mass transfer limitations, which produce solute gradients through the spheres. Therefore, only the outer areas of the often-intricate networks formed are physiologically active, resulting in highly viscous culture broths and nutrient limitation in the centre of the pellet (Figure 3). These properties are unfavourable for biotechnological applications (Hobbs *et al,* 1990).

A fermentation process is commonly employed by researchers in order to cultivate microorganisms and scale-up primary and secondary metabolite production. In fed-batch systems the culture goes through a lag and an exponential phase before it ultimately reaches the stationary phase. The fed-batch system often involves the addition of specific precursor compounds, usually amino acids, in order to facilitate a particular biosynthetic pathway in an attempt to enhance and direct the manufacture of desired products (Meiwes *et al*., 1990). There is evidence that some actinomycetes prefer metabolising certain amino acids than refined sugars (Hoskisson *et al*., 2003).

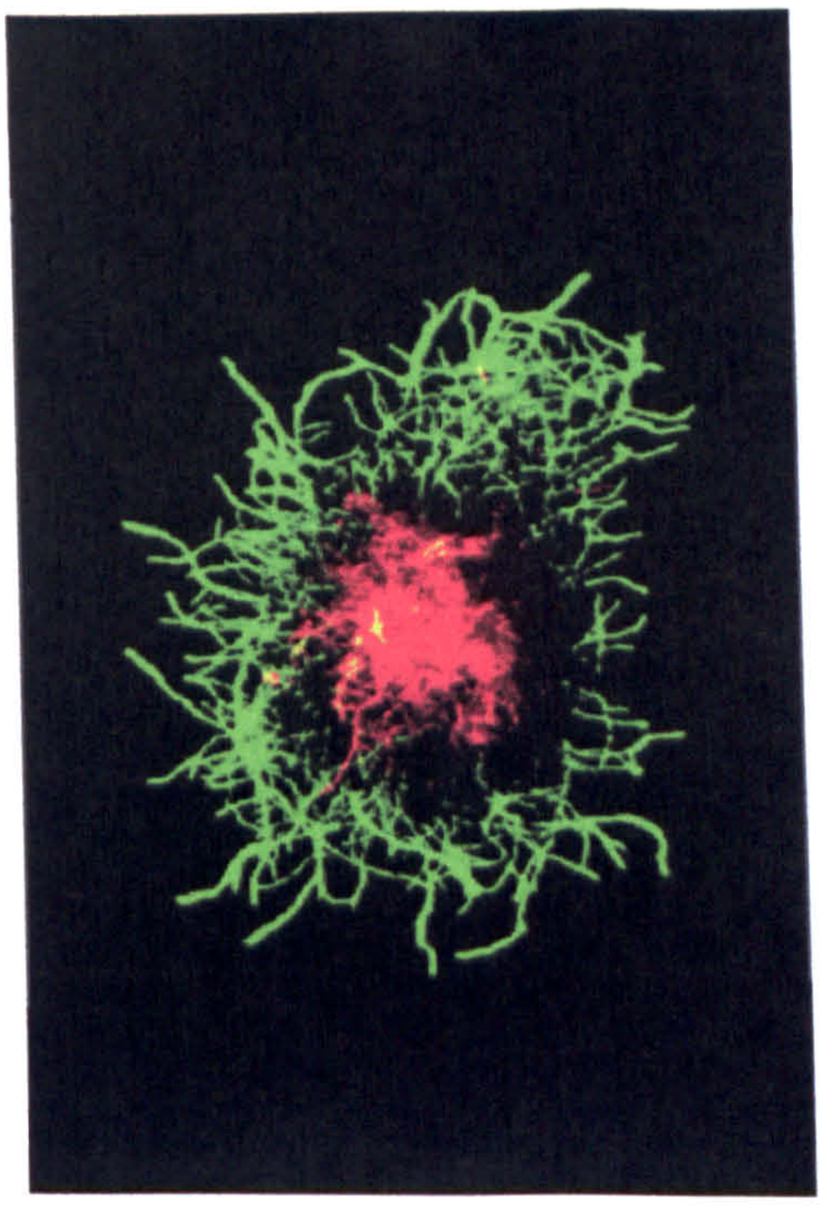


Figure 3: A pellet of *S. coelicolor* grown in chemostat culture at low dilution rate (0.02 h^{-1}). The pellet was stained using BacLight (Cell Viability Kit; Molecular Probes) and the image collected by deconvolution microscopy. The green areas of the pellet indicate hyphae with intact functioning membranes; the red areas are those with compromised membranes (Redrawn from cover picture in *Microbiology*, 151, 3147-3346).

1. 1. 3. *Streptomyces coelicolor*

In 2002 the *S. coelicolor* genome was published (Figure 4). The 8,667,507 base pair linear chromosome of this organism contains the largest number of genes so far discovered in a bacterium. The origin of replication *(oriC)* is centrally located and the terminal inverted repeats (TIRs) carry covalently bound protein molecules on the free 5' end. Replication occurs bidirectional from *oriC*, leaving a terminal single- stranded gap on the discontinuous strand, which is filled by DNA synthesis primed from the terminal protein (Bentley *et al.*, 2002, http://www.sanger.ac.uk/Projects/S_coelicolor/).

General characteristics of the chromosome sequence are shown in Table 1. The *oriC* and *dnaA* gene lie about 61 kb left of the centre, at 4,269,853- 4,272,747bp. Moreover, there is a slight bias (55.5%) towards coding sequences on the leading

strand and GC bias is decreased around *oriC*. The way different types of genes are distributed reveals a central core occupying half of chromosome and a pair of chromosome arms. Essential genes, such as those for cell division, DNA replication, amino acid biosynthesis, translation and transcription are found in the core. Non-essential genes, such as secondary metabolites, hydrolytic exoenzymes and 'gas vesicle' proteins are located in the arms of the chromosome.

Component of chromosome	Property
Total size	8,667,507bp
Terminal inverted repeat	21,653bp
G+C content	72,12 %
Coding sequence	7,825
...of which pseudogenes	55
Coding density	88,9 %
Average gene length	991bp
Ribosomal RNAs	6 x (16S-23S-5S)
Transfer RNAs	63
Other stable RNAs	3

Table 1: General features of *S. coelicolor* chromosome. Redrawn from Bentley *et al.*, 2002.

The 7,825 predicted genes include more than 20 clusters coding for known or predicted secondary metabolites (Table 2). Two clusters SCO0492 and SCO7681-7683 direct the biosynthesis of the novel siderophores coelichelin and coelibactin, whereas a third one SCO2782-2785 regulates the production of desferrioxamines G1 and E. The fact that *S. coelicolor* needs three clusters encoding siderophore production implies that the strain is under pressure to compete in nature and take up iron. The biosynthesis of these siderophores is encoded by classical non-ribosomal peptide synthases (NRPSs).

The genome includes 965 regulatory proteins (12.3 %), of which 819 (10.5 %) are thought to be secreted ones valuable for scavenging nutrients from the environment. It also has 614 proteins with predicted transport function, including iron acquisition. Moreover a group of 25 putative DNA-binding proteins have been identified and may constitute a new *Streptomyces* specific family of regulators.

Biosynthetic system	Metabolite	Size, kb	Location
Type II PKS	Actinorhodin	22	5071–5092
Type II PKS	Gray spore pigment	8	5314–5320
Mixed	Methylenomycin	20	SCP1 plasmid
NRPS; type I modular PKS	Prodiginines	33	5877–5898
NRPS	CDA	80	3210–3249
NRPS	Coelichelin	20	0489–0499
NRPS	Coelibactin	26	7681–7691
NRPS	Unknown	14	6429–6438
Type I modular PKS	Unknown	70	6273–6288
Type I modular PKS	Unknown	10	6826–6827
Type I iterative PKS	Polyunsaturated fatty acid?	19	0124–0129
Chalcone synthase	Tetrahydroxynaphthalene	1	1206–1208
Chalcone synthase	Unknown	3.5	7669–7671
Chalcone synthase	Unknown	1	7222
Sesquiterpene synthase	Geosmin	2	6073
Sesquiterpene synthase	Unknown	2.5	5222–5223
Squalene-Hopene cyclase	Hopanoids	15	6759–6771
Phytoene synthase	Isorenieratine		0185–0191
Siderophore synthetase	Desferrioxamines	5	2782–2785
Siderophore synthetase	Unknown	4	5799–5801
Type II fatty acid synthase	Unknown	10	1265–1273
Butyrolactone synthase	Butyrolactones?	1	6266
Deoxysugar	Unknown	20	0381-0401

Table 2: Gene clusters potentially directing the production of secondary metabolites in *S. coelicolor*. Redrawn from Challis & Hopwood, 2003.

Using silver staining Hesketh and co-workers successfully detected more than 40 % of the genome's proteins. Nevertheless no proteins were detected from the metabolic clusters of coelichelin and coelibactin, suggesting that the conditions might not have promoted the siderophore production (Hesketh *et al.*, 2002). In a second attempt in 2005 they identified predicted lipoproteins and ABC transporters that might regulate iron binding, acquisition and transportation (Kim *et al.*, 2005).

Streptomycetes are members of Actinomycetales, which include *M. tuberculosis*, the causative agent of tuberculosis, and *M. leprosy*, the causative agent of leprosy, genomes of which have been sequenced as well. *S. coelicolor*'s genome poses great similarities with these genomes and considerable inferences can be drawn as a result of genome-comparisons between pathogenic and non-pathogenic members of the family.

Last but not least over two thirds of the naturally excreted antibiotics currently in use are produced by streptomycetes and numerous previously uncharacterised enzymes playing a crucial role in the production of natural products, have become a resource of vast potential value. Understanding and investigating these enzymes will facilitate the genetic manipulation of mechanisms to produce or over-produce new compounds with potential therapeutic and antimicrobial activity (Bentley *et al.*, 2002, http://www.sanger.ac.uk/Projects/S_coelicolor/).

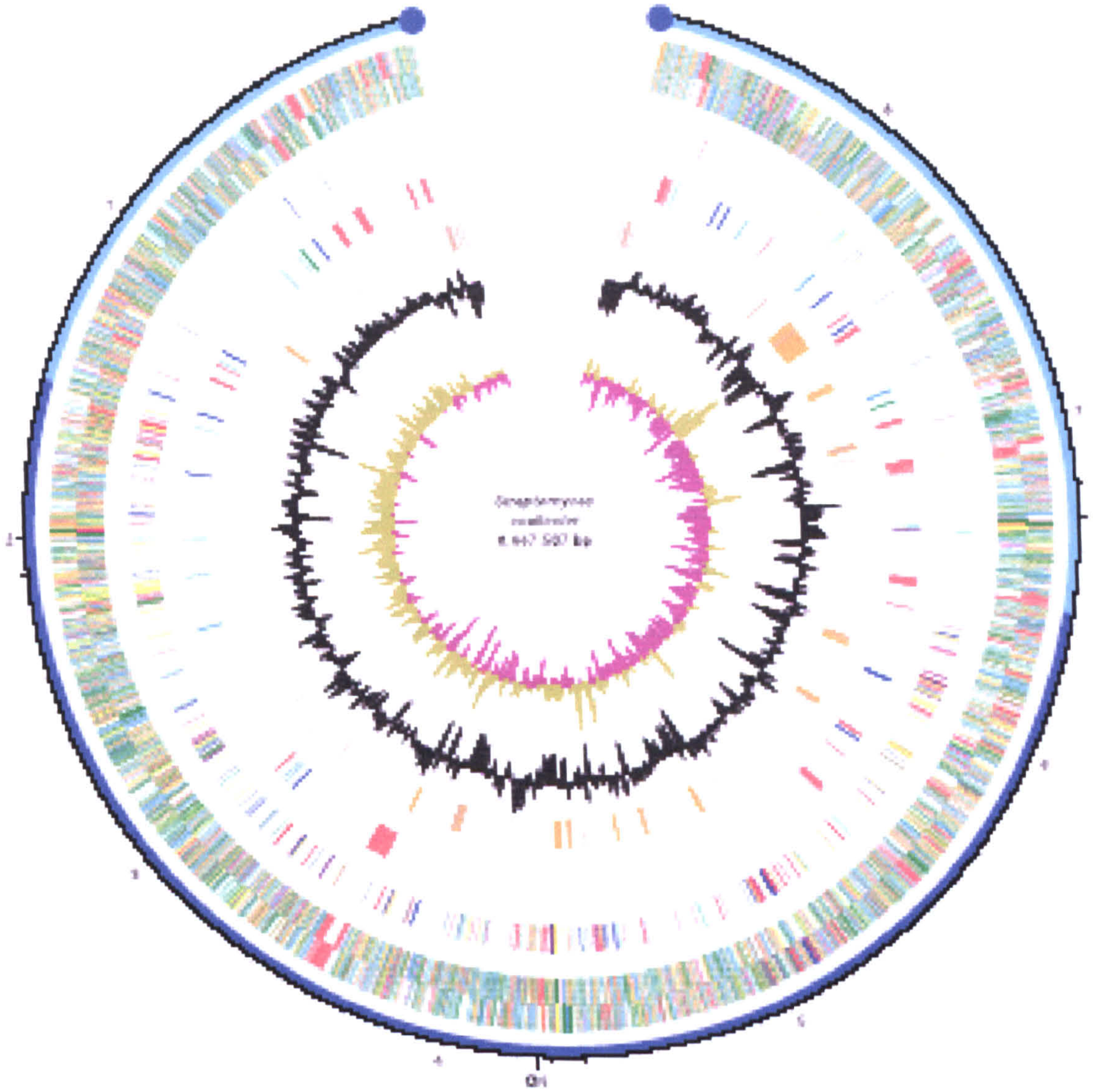


Figure 4: Circular representation of the *S. coelicolor* chromosome. The outer scale is numbered anticlockwise in megabases and indicates the core (dark blue) and arm (light blue) regions of the chromosome. Circle I and 2 (from the outside in): all genes (reverse and forward strand, respectively) colour-coded by function (black-energy function; red-information transfer and secondary metabolism; dark green-surface associated; cyan-degradation of large molecules; magenta- degradation of small molecules, yellow-central and intermediary metabolism; pale blue-regulators; orange-conserved hypothetical; brown-pseudogenes; pale green-unknown; grey-miscellaneous. Circle 3: selected 'essential' genes (for cell division, DNA replication, transcription, translation and amino-acid biosynthesis, colour-coding as for circles I and 2). Circle 4: selected 'contingency' genes (red- secondary metabolism; pale blue-exoenzymes; dark blue-conservon; green-gas vesicle proteins). Circle 5: mobile elements (brown-transposases; orange-putative laterally acquired genes). Circle 6: G+-C content. Circle?: CC bias ((G-C/G+C), khaki indicates values>l, purple<l). The origin of replication *(oriC)* and terminal proteins (blue circles) are also indicated. Redrawn from: http://www.sanger.ac.uk/Projects/S_coelicolor/Sco_Nature.pdf.

1. 2. 0. Iron

Iron is an essential, particularly scarce and potentially hazardous entity in nature. Microorganisms growing under aerobic condition need iron for vital cellular functions including DNA replication and reduction of oxygen for ATP synthesis; but when in excess iron initiates lethal reactions.

In the presence of oxygen, at physiological pH, iron forms insoluble ferric complexes thus decreasing this ion's availability to microorganisms. Bacterial iron metabolism involves the transcription of genes responsible for the acquisition, transport and storage (Wandersman & Delepelaire, 2004). Failure to control the above mechanism results in iron deficiency or conversely iron overload. At least 1μM of iron is needed for optimum bacterial growth. It should be mentioned that not all bacteria require iron. The genus of *Lactobacillus* is probably the only exception where iron does not facilitate growth, as there is no heme or other iron containing enzymes. In higher organisms it remains in association with transferrin (Tf), lactoferrin (Lf) and ferritin, which are transport and storage proteins. Tf is never fully saturated with iron so that in the case of infection it can take up any free iron circulating in the blood (Neilands, 1995 and Wandersman & Delepelaire, 2004). Tfs are also found in plants (phytoferritins) and in some microorganisms (bacterioferritins). The latter Tfs are sometimes found in fungi and differ from animal and plant Tfs as they are attached to a heme group (Ratledge & Dover, 2000).

Iron is related to some of the most ancient therapies applied by man. In 1500BC Melampus treated Iphyclus, prince of Thessalia (Greece), for his sexual impotance by scraping the rust of an old knife into his wine. Iron was also used as a cure for chlorosis since the 17th century. Last but not least Bland's pills, which contain ferrous sulphate, were used for anaemia treatment since 1831 (Crichton & Pierre, 2001).

1. 2. 1. Copper

Copper is found in the earth's crust at 55 ppm. Following Fe and Zn it is the third most abundant transition element in the body. A healthy human body should contain 50-120 mg of copper. This element is distributed along the body, including the heart, liver, lungs, spleen and brain (Alexandrova *et al.*, 2003 & Crichton & Pierre, 2001). Although trace amounts of copper are essential for life, excess of the element is toxic. It is not yet clear if environmental levels of copper pose a health risk to humans; however when copper above 25 mg is taken orally the symptoms are nausea, diarrhoea, vomiting and intestinal cramps. These symptoms start at blood concentrations of 3 mg Cu/L. In severe intoxication, after exposure to copper sulphate sprays, lung tissue damages occur and furthermore copper dust cause decolourisation of the skin. Copper sulfates cause gastrointestinal tract damages and a 5% w/v solution is regularly used as an emetic agent after poisoning.

Copper therapy is ancient and it was practiced 1550 years Before Christ, in the middle Empire in Egypt. It is first mentioned as a medical treatment (anti-inflammatory agent) in Papyrus Ebers, where doctors attempted to master disease by magic and therapy (Alexandrova *et al.*, 2003 & Crichton & Pierre, 2001).

1. 2. 2. Iron and copper evolution

Soluble ferrous iron was available during prebiotic times and it was the type used in the first stage of life. According to the chemoautotrophic theory, the origin of life was created in an iron-sulphur based world. The gases CO and CO_2 were produced by volcanoes and hydrothermal vents. The reductive formation of CH_3SH and its carbonylation into activated thioester CH_3COSCH_3 played the role of acetyl-CoA. The energy for this reaction was retrieved from the oxidation of iron pyrite from iron sulphide and hydrogen sulphide, which provided a foundation for all the chemical conversions (Crichton & Pierre, 2001).

The evolution of the di-oxygen (O_2) in the earth's atmosphere began to develop 10^9 years ago when prokaryotic life was expanding. There was a lag phase of 200-300 million years between the first production of O_2 and its establishment in the environment since O_2 was utilised for the oxidation of ferrous ions in the oceans. The introduction of O_2 had a disastrous effect on most microorganisms and in many cases it is considered as the first instance of pollution on the earth (Crichton & Pierre,

2001). As a consequence iron was oxidised into an insoluble form and bioavailability of iron decreased.

On the contrary, the oxidation of insoluble copper (I) led to the soluble form of copper (II). Proteins and enzymes involved in the anaerobic metabolism were not suitable for the di-oxygen environment and therefore copper began to be utilised in energy-acquisition systems such as cytochrome oxidase (for a review see Crichton & Pierre, 2001).

In nature there were two possible scenarios: either use insoluble iron with adaptation to the dioxygen availability or begin utilising soluble copper. Both of them occurred evolutionarily. Microorganisms that survived had to adapt to the biotransformation and develop mechanisms for incorporating copper into enzymatic reactions and solubilising and acquiring iron. The latter situation is where siderophores where believed to have evolved (Cox, 1994).

1. 3. 0. Siderophores

Siderophores, from the Greek word iron carriers, are low molecular weight compounds (600-1500 Daltons) that chelate the ferric complexes and other ions with a high affinity (Neilands, 1995). These include Fe (II), Mn(II), Zn(II), Cu(II), Co(II), Ni(II), Mg(II), Al (III), Ga(III), Cr(III), In(III), Sc(III) [John *et* al., 2001, Kalinowski *et al.*, 2000 and Rodriguez & Smith, 2003]. Siderophore production is restricted to bacterial and fungal species. They are produced under iron-limited conditions in order to capture insoluble iron from the environment and make it available to the cell. These compounds are mainly divided into two chemical groupings, hydroxamates and catechols, and they are classified on the basis of the chemical functional groups that they use to chelate iron.

1. 3. 1. Hydroxamates

In the hydroxamate siderophores, nitrogen atoms of thiazoline rings are used to chelate ferric ion (Crosa & Walsh, 2002). Desferrioxamines (A_1, A_2, B, C, D_1, D_2, E, F and G) represent this category and are produced by a number of species of *Streptomyces*, *Nocardia*, *Micromonospora*, *Arthrobacter*, *Chromobacterium* and *Pseudomonas* (Figure 5). *Streptomyces pilosus* excretes all the above-mentioned types of desferrioxamines; however B is the main group. Ferrioxamines are structurally similar and their characteristic is the repeating units of α-amino-ω-hydroxyaminoalkane and succinic or acetic acid. There is only a difference on a side

chain between B (linear) and D_1 (linear): the amino group of B is acetylated in D_1. Moreover the difference between the cyclic ferrioxamines B and D_2 is that the 1-amino-5-hydroxyaminopeptane of E is replaced with a 1-amino-5-hydroxyaminobutane (Muller & Raymond, 1984 and Gledhill, 2001).

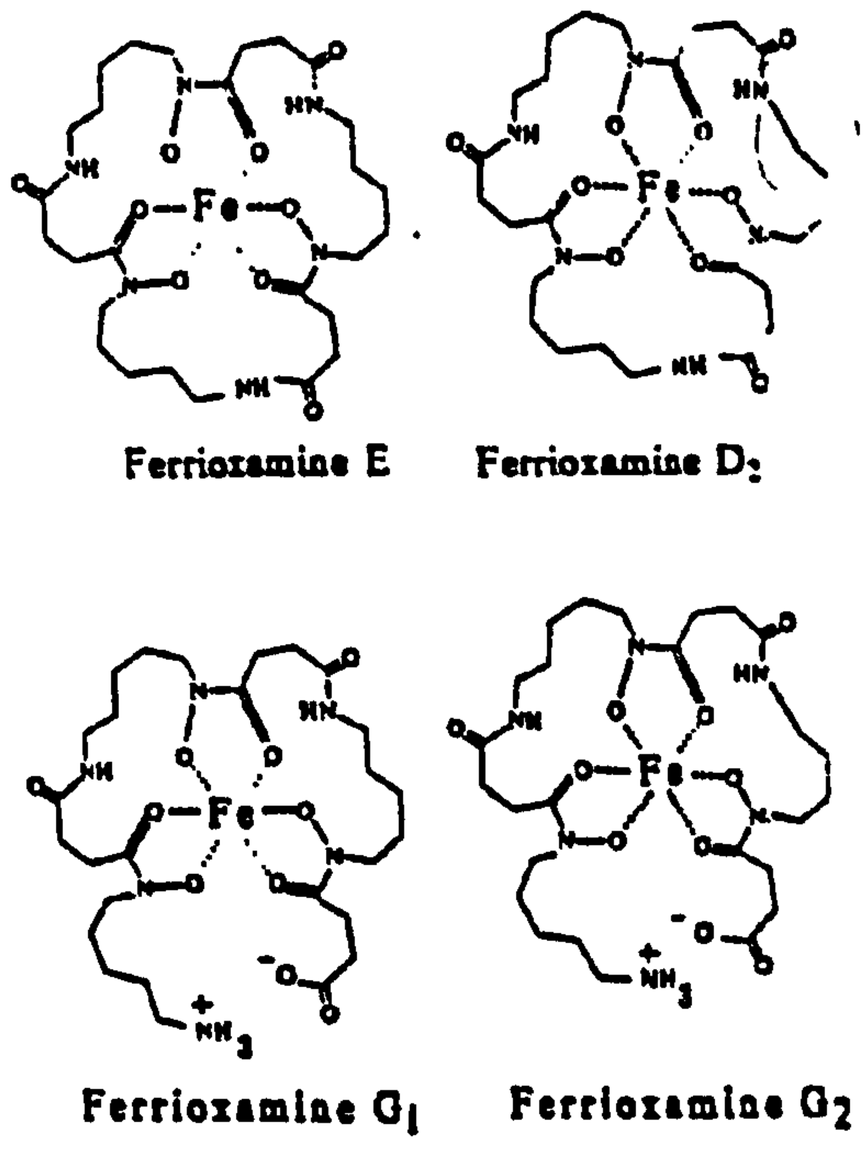


Figure 5: Structures of the cyclic ferrioxamines E and D_2 and the corresponding linear ferrioxamines G_1 and G_2. Redrawn from Reissbrodt *et al.*, 1990.

Rhizobia are nitrogen-fixing bacteria well known to produce a wide variety of siderophores, especially hydroxamates, such as anthranilate, rhizobactin, rhizobactin 1021 (dihydroxamate), vicibactin (trihydroxamate) and vicibactin 7101. In general production in Rhizobia is species specific: *Sinorhizobia* produce di-hydroxamates, *Mesorhizobia* produce trihydroxamates and *Bradyrhizobia* produce neither of them (Carson *et al.* 2000). The role of siderophores in Rhizobia is vital, as they need iron to convert molecular nitrogen to ammonia (Figure 6); moreover there is evidence that some of the rhizobia siderophores have antimicrobial activity against other plant pathogens (Jadhav & Desai, 1992).

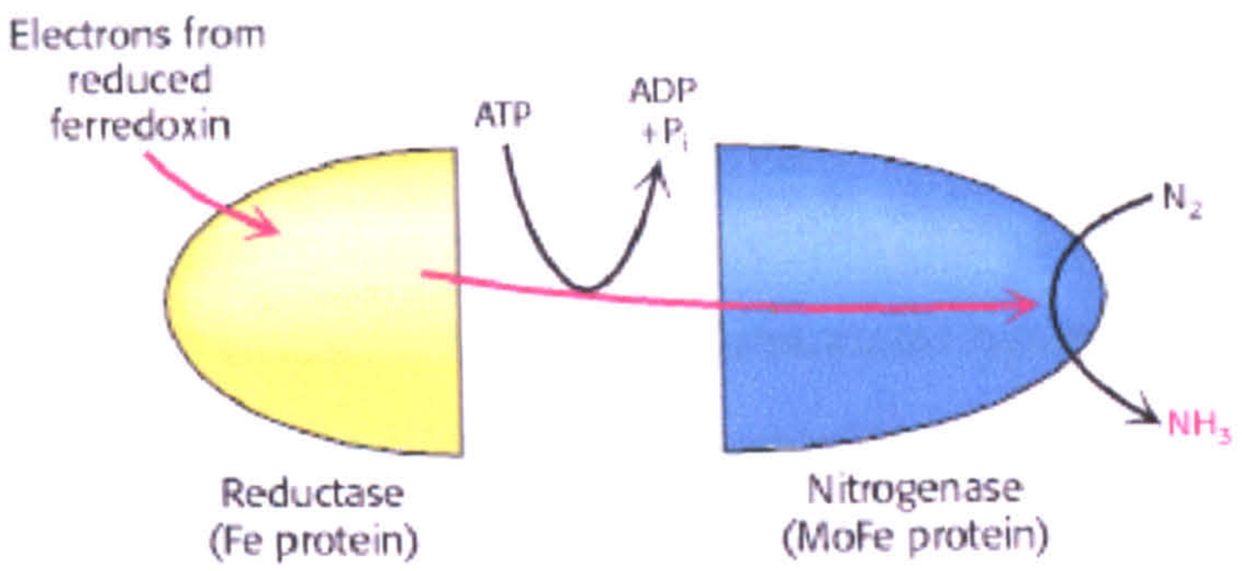


Figure 6: Nitrogen fixation by Rhizobia species where electrons flow from ferredoxin to the reductase (iron protein, or Fe protein) to nitrogenase (molybdenum-iron protein, or MoFe protein) to reduce nitrogen to ammonia. ATP hydrolysis within the reductase drives conformational changes necessary for the efficient transfer of electrons.

Redrawn from Berg *et al.*, 2002. Also available online: http://www.ncbi.nlm.nih.gov/books/bv.fcgi?call=bv.View..ShowSection&rid=stryer.section.3337.

Aerobactin is commonly produced by *Escherichia*, *Salmonella*, *Klebsiella* and *Shigella* species. However *Aerobacter aerogenes* 62-I is considered to be the main strain producer (Neilands & Nakamura, 1991).

Ferrichrome siderophores are cyclic hexapeptides produced by a variety of fungi including *Ustilago*, *Aspergillus*, *Neovossia* and *Penicillium* species (Muller *et* al., 1984 and Konetschny-Rapp *at al.*, 1988). Furthermore an alanine-conjugate ferrichrome has been isolated from a yeast species, *Cryptococcus melibiosum* (Atkin *et al.*, 1970). Unfortunately this organism has not been studied further (for more details about ferrichrome siderophores please refer to xenosiderophores section).

In a siderophore screening programme, which included 142 strains of 19 genera of yeasts or yeast-like organisms it was demonstrated that rhodotorulic acid, a dihydroxamate ornithine conjugate, is excreted by at least 52 strains including strains from *Aessosporon* (3 strains), *Cryptococcus* (1 strain), *Leucosporidium* (3 strains), *Rhodosporidium* (4 strains), *Rhodotorula* (27 strains), *Sporidiobolus* (2 strains) and *Sporobolomyces* (12 strains) [Atkin *et al.*, 1970 and Muller *et al.*, 1984].

1. 3. 2. Catechols

Catechols bind iron by using the hydroxyl groups of catechol rings, which normally consist of di-hydroxybenzoyl (2, 3-DHBA) conjugates. Enterobactin, also known as enterochelin, is a tri-ester ring that contains three side chains of 2, 3-DHBA serine (Figure 7); it is produced in *Escherichia coli*, *Salmonella typhimurium* and some nitrogen-fixing bacteria such as *Klebsiella pneumoniae* and *Klebsiella terrigena* (Hofte, 1993 & Payne, 1994).

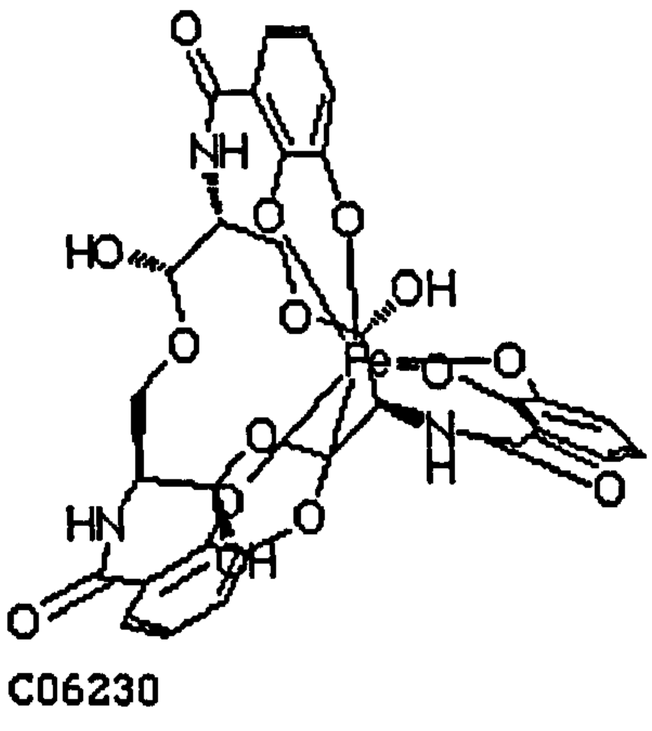


Figure 7: Ferric form of enterobactin. Redrawn from Payne, 1994.

A threonine conjugate of 2,3-DHBA is produced by *Klebsiella oxytoca* and *Escherichia* species (yield 32 mg/L) as well and a glycine-2, 3-DHBA has been isolated from strains of *Bacillus*. A diphenolic acid conjugate of lysine has been detected in cultures of *Azotobacter vinelandii* and *Rhizobium* RA-1 (yield 6.2 mg/L) produces a catechol, which represents a threonine, glycine-2, 3-DHBA. *Agrobacterium tumefaciens* produces a threonine-spermidine-2, 3-DHBA (yield 6-9mg/L), called agrobactin (Mayuranki *et al.* 1985). An alanine-lysine-2, 3-DHBA has been identified in cultures of Cowpea *Rhizobium* species isolated from the peanut plant, *Arachis hypogea*. Addition of the two amino acids (concentration of 20 mM) in the media led to fourfold improvement of growth (Jadhav & Desai, 1992). Moreover it was demonstrated that not only the catechol binds to molybdenum but the later regulates the biosynthetic pathway of the chelatimg agent as well. Addition of 0-1 mM molybdenum led to an increase of catechol production, whereas addition of the ion above that concentration resulted in a decrease of siderophore excreted (Figure 8). Amending the media with 10 μM $Fe_2(SO_4)$ stimulated molybdenum uptake by 318 %, which was further increased by 100 % when 33μg of the siderophore were also present (Pater *et al.*, 1988).

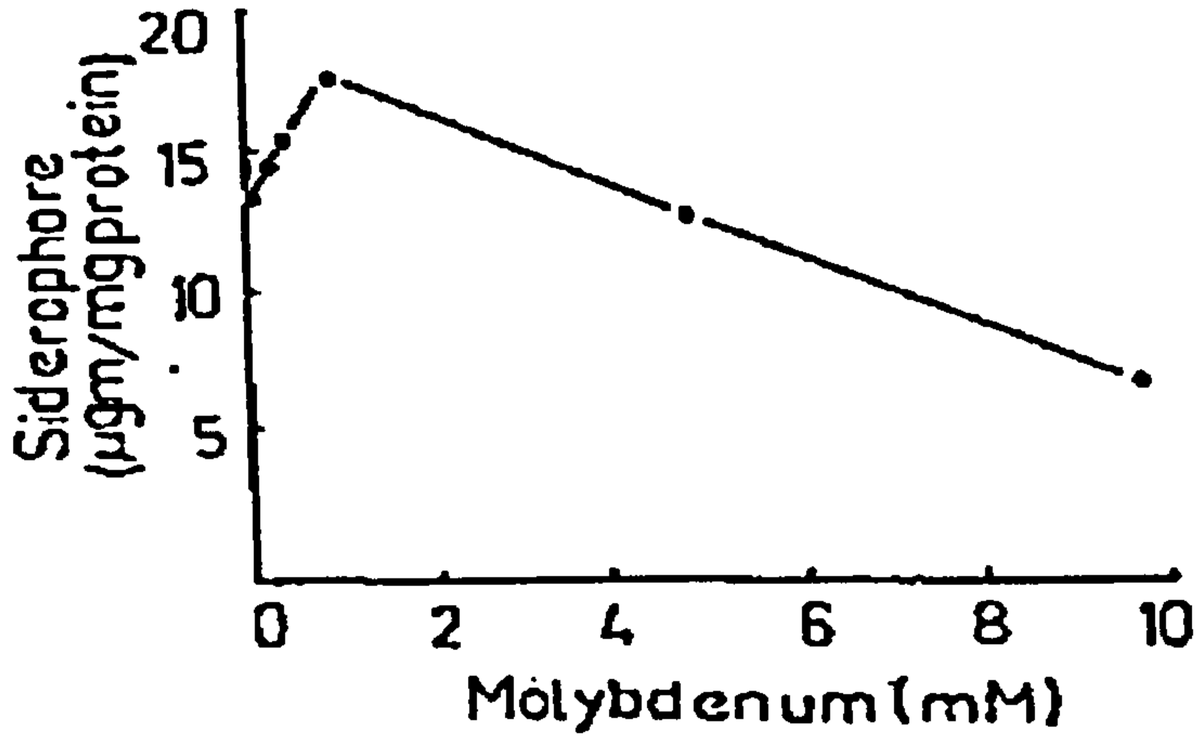


Fig 8: The siderophore production of *Rhizombium* sp. in the presence of increasing concentrations of molybdenum. Redrawn from Patel *et al.*, 1988.

Brucella abortus produces a highly efficient iron capturing catecholic compound, which has been identified as 2, 3-dihydroxybenzoate (2, 3-DHBA). Although its role as a bacterial siderophore has been criticised (Chipperfield & Ratledge, 2000), in 2002 the substance was recognised as a siderophore, brucebactin. There is not sufficient evidence that *B. abortus* produces any other type of siderophores (Gonzalez-Carrero *et al.* 2002).

Cyanobacteria, the prototype siderophore producers in the marine environment, are well known to produce iron-chelating agents in their alkaline habitat, where the bioavailability of iron is very low. For many years they were considered as hydroxamate - producers only and schizokinen, excreted by *Anabaena* ATCC27898, is still the only structurally characterised hydroxamate synthesised by a cyanobacterium. However in 1994 it was discovered that not only certain cyanobacteria, such as *Synechococcus* species, produce catechol compounds but also multiple siderophore production is very common amongst the group (Table 3) [Wilhelm & Trick, 1994].

Furthermore *Rhodomonas ovalis* was found responsible for the siderophore production in marine eukaryotic phytoplankton. The structure has not been characterised yet but it appears to play a crucial role in the carbon cycle of the earth (Kanako *et al.* 2001).

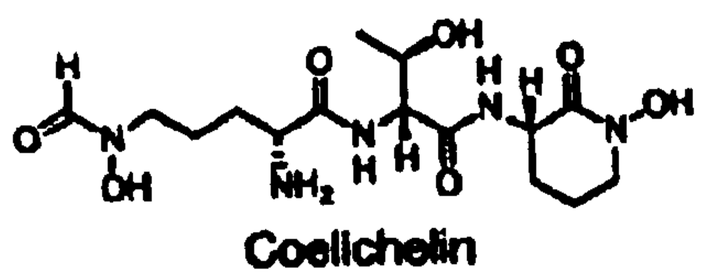
Strains	Siderophores	Hydroxamates	Catechols
Freshwater			
Synechococcus sp. PCC6031	3	2	1
Synechococcus sp. PCC 6908	2	1	1
Synechococcus sp. PCC 7942	1	1	-
Synechococcus sp. PCC6803	1	-	-
Oscillatoria tenuis Ag. UTEX 428	3	1	2
Anabaena catenula UTEX375	2	1	1
Marine			
Synechococcus sp. PCC 7002	4	1	2
Synechococcus sp. WH 8101	2	1	1
Synechococcus sp. WH 7805	1-2	ND	ND
Synechococcus sp. BBC2A	2	ND	ND

Table 3: Production of extracellular siderophores by iron-limited cultures of cyanobacteria (ND-Not determined). Redrawn from Wilhelm & Trick, 1994.

1. 4. 0. Multiple siderophore production

Members of streptomycetes are well acknowledged for their ability to produce multiple siderophores, which are independently regulated and act contingently in order to compete more efficiently in their environment. *S. coelicolor* is known to have the capacity to produce desferrioxamine E and G1 as well as coelichelin and coelibactin (Figure 9). There is evidence that although the siderophores are structurally distinct their biosynthesis is coregulated. For example both *cch* (coelichelin) and *des* (desferrioxamine) clusters responsible for the production of coelichelin and desferrioxamines respectively, are not closely linked on the *S. coelicolor* genome but do contain similar inverted repeat sequences that match the IdeR binding (further explanation of the molecular iron regulation follows in a future section). Moreover an ironbound lipoprotein has been found on both clusters,

indicating that the organism selectively co-regulates the two independent pathways (Challis & Ravel, 2000).



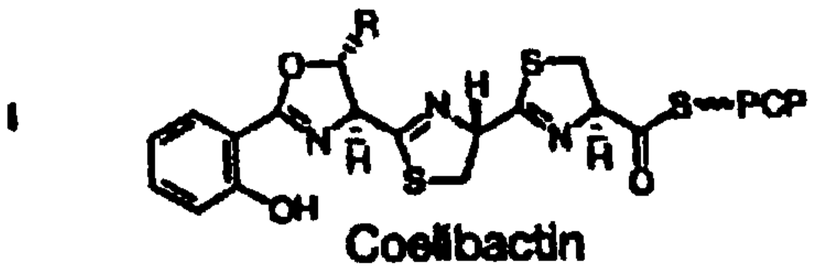


Figure 9: Structures of the siderophores coelichelin and coelibactin produced by *S. coelicolor*. Redrawn from Bentley *et al*, 2002.

This mechanism can be explained by the fact that there are numerous organisms, which cannot synthesise desferrioxamines themselves but can utilise them as xenosiderophores. In order for *S. coelicolor* to overcome this problem and out compete in its natural environment, it has evolved a second cluster of genes directing a different type of siderophore (coelichelin), which when bound to iron will be recognised by the cell via an independent transport system from that of ferrioxamine uptake. In this way the chances of desferrioxamine piracy are lessened. This is a paradigm of two structurally different compounds acting contingently as opposed to the classical synergetic behaviour of secondary metabolites (Challis & Hopwood, 2003).

S. tendae Tu 901/8c produces enterobactin as well as a desferrioxamine B and E. Moreover *S. pilosus* can excrete, under iron-deficient conditions, various desferrioxamines including A_1, A_2, B, C, D_1, D_2, E, F and G (Muller & Raymond, 1984). Furthermore *S. olivaceous* TU 2718 is known to produce both desferrioxamines E and D_2 (Meiwes *et al.*, 1990).

Salmonela enteritica, a uropathogenic strain, produces two catechol-like compounds, enterobactin and salmochelin when grown in iron deficiency. Salmochelin S4 is the major one and is described as a doubly-glycosylated enterobactin derivative (Zhu *et al.*, 2005).

Aspergillus ochraceous is also a paradigm of multiple siderophore production. All of them belong to the ferrichrome family and include ferrirubin and ferrichrysin, the predominant and second major siderophore respectively (62% and 15% of the total siderophores). Moreover the strain synthesises some other chelating agents called asperchromes A, B1, B2, C, D1, D2, D3 and fusigen (Jalal *et al.*, 1984).

Oscillatoria temuis Ag. UTEX 428, a cyanobacterium, produces two catechols and one hydroxamate (Wilhelm & Trick, 1994). Production of the siderophores in that strain is regulated by different amounts of iron availability. In concentrations of iron of 4.1×10^{-7} M $FeCl_3$ and above the isolate produces a hydroxamate-type siderophore, whereas a catechol-like one is produced at lower levels. It is also known that production of catechol enhances maximal growth rate, whereas cells maintain lower growth rates when hydroxamate is synthesised. To date it is not known why cyanobacteria produce multiple classes of siderophores; but it has been proposed that organisms might need more than one siderophore in order to survive under different conditions. When the first one fails to be excreted a second one, possibly a stronger chelator, is induced. In general catechols are very stable in alkaline conditions making them the predominant siderophore in marine environments (Brown & Trick, 1992).

A similar mechanism has been developed by the fungus *Ustilago sphaerogena*, which produces two different hydroxamates (ferrichromes). When the organism is unable to synthesize ferrichrome, it triggers the production of ferrichrome A, a more powerful chelating agent. *Fusarium roseum* also behaves similarly by producing both the ester-type fusarinines and the peptide desferrimalonichrome (Ecker *et al.* 1982).

Hafnia alvei, previously named *Enterobacter hafniae*, and *Enterobacter agglomerans*, previously named *Erwinia herbicola* belong to Enterobacteriaceae family and they are genetically closely related. However *Hafnia alvei* produces the linear hydroxamates G_1 and G_2 whereas *Enterobacter agglomerans* produces the cyclic versions, E (main) and D_2. This indicates that although ferrioxamines are mainly found in gram-positive bacteria, gram-negative enterobacteria can also synthesize and transport ferrioxamines (Reissbrodt *et al.* 1990).

Erwinia chrysanthemi 3937, a plant pathogen, produces two catecholic compounds. Chysobactin is a monomer of 2,3-dihydroxybenzoyl-$_D$-lysyl-$_L$-serine derivatives. Achromobactin belongs to the hydroxyl/carboxylate class [Rauscher *et al.* 2002].

E. coli A36 is a bovine pathogen that causes diarrhoea and produces both the catecholic compound, enterobactin or enterochelin, and the hydroxamate, aerobactin. The latter is a plasmid encoded compound related to the strains virulence. It has been

proposed that the two catecholic compounds behave antagonistically. Although aerobactin portrays a lower affinity for iron, it is the dominant siderophore formed during bacterial growth. Both iron chelators are produced at the same time but enterochelin is stored in the periplasm before a late excretion occurs. The ability of enterobactin to scavenge iron was confirmed by the fact that aerobactin-deficient mutants were still able to chelate iron. Furthermore both aerobactin deficient mutants and wild type cells secreted enterobactin at the same time. This suggests that the aerobactin's superiority is due to the late release of the agent and not the antagonism between the two (Der Vartanian, 1988).

Rhodococcus erythropolis IGTS8 is reported to produce two new types of siderophores called heterobactin A and B. They are both tripepides representing $_{L}$-ornithine-glycine-$_{D}$-ornithine conjugates of DHBA. This is the first description of iron chelating compounds excreted by rhodococci, which are phylogenetically related to Mycobacteria and Nocardia species. *R. erythropolis* IGTS8 has commercial application in biodesulfurization of fuel oil. The strain removes up to 11% of petroleum and coal fuel sulphur that when combusted produce sulphur dioxide, the main component of acid rain. The organism exhibits a long lag phase in standard media thought to be due to the inhibitory effect of the iron present in the medium. It has been suggested that, in order to achieve a commercial viable microbial process, the option of cultivation in iron-deficient media needs to be explored (Carrano *et al.* 2001) under these conditions the production of siderophores is essential to the growth of the organism.

1. 5. 0. Xenosiderophores

It has already been mentioned that *S. pilosus* can synthesize under iron-deficiency the desferrioxamines A_1, A_2, B, C, D_1, D_2, E, F and G. Investigation of iron transport using ^{55}Fe has revealed that the strain can pirate iron from ferri -complexes of B, D_1, D_2, and E when supplied in a concentration of 2 μM. The organism can also take up ^{67}Ga and chromium (III) from the siderophores at similar rates as ^{55}Fe (Table 4) [Muller & Raymond, 1984].

Time (min)	Uptake (nmol/mg [dry weight] of cells of:	
	Cr (III)	^{55}Fe
30	2.00	1.73
60	3.10	2.65
90	3.90	3.40

Table 4: Uptake of ^{55}Fe and Cr (III) desferrioxamine B determined by atomic absorption. Redrawn from Muller & Raymond, 1984.

Moreover there is evidence that *S. pilosus* can accrue iron from exogenous ferrichrome siderophores (ferrichrome, ferrichrysin, ferrirubin, ferrichrome B), rhodotorulic acid (RA) and synthetic enantio-RA (Figure 10). Ferrichrome and ferrichrysin, which have different amino acid sequences, are acquired by the strain at a similar rate, which is 15 % more than that of ferrioxamine B's and RA's. Nevertheless ferrirubin, which is structurally very similar to ferrichrysin, was far less efficient (80 %) at providing iron to the strain compared to ferrichrysin uptake. Surprisingly the synthetic RA was twice as efficient in providing iron to the strain as the natural RA. Uptake of all the *S. pilosus* xenosiderophores mentioned above is inhibited by chromic desferrioxamine B, indicating that in this organism there is only one ion transportation system (Muller *et al.*, 1984).

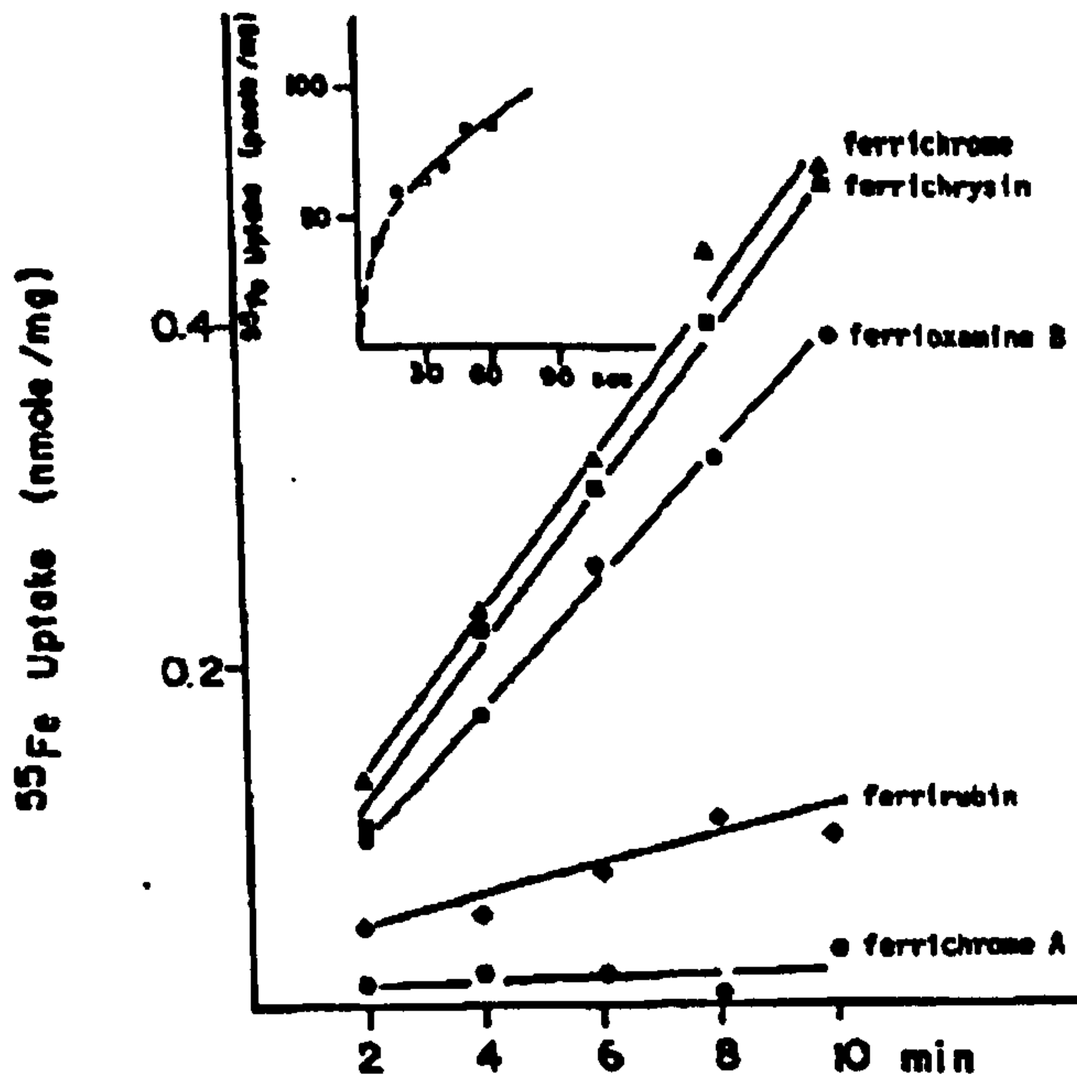


Figure 10: Time-dependent uptake of ^{55}Fe from ferrichrome siderophores and ferrioxamine B at 1 μM concentration. Redrawn from Muller *et al.*, 1984.

Recently it has been published that a compound from *S. griseus* stimulates the growth, aerial mycelium formation, yellow and melanin-like pigment and antibiotic production of *S. tanashiensis*. Further investigation revealed that the substance inducing the colonial development and secondary metabolism of *S. tanashiensis* is desferrioxamine E. Although the strain is unable to synthesize the siderophore it has specific binding proteins for taking it up as a xenosiderophore. The results were verified by adding purified desferrioxamine E in a *S. tanashiensis* culture. Desferrioxamine B had little effect on stimulating the isolate's growth. The exogenous supply of other siderophores, such as ferrichrome, transferrin, nocobactin, did not influence *S. tanashiensis* phenotype. Moreover disruption of the biosynthetic gene cluster responsible for desferrioxamine E production in *S. coelicolor* A3(2) resulted in a mutant, KYI, unable to synthesise the siderophore or promote *S. tanashiensis*'s growth. The KYI strain demonstrated impaired growth, which was partially, but remarkably, restored by addition of exogenous desferrioxamine E. Partial restoration can be easily explained by the presence of a second siderophore, coelichelin (Yamanaka *et al.*, 2005). The case of *S. tanashiensis* not only verifies that siderophores play a crucial role in iron acquisition but also provides, to our knowledge, the first evidence of the relationship between growth and siderophore production in streptomycetes.

S. viridosporus is a multiple siderophore producer synthesising both desferrioxamine B and E. Nonetheless it is able to scavenge iron from ferrioxamine G when supplied exogenously. Furthermore despite the fact that *S. lividans* cannot excrete desferrioxamine B it has been demonstrated that it can acquire iron from it as efficiently as *S. viridosporus* (Imbert *et al.*, 1995).

Although *E. chrysanthemi* 3937 is unable to produce enterobactin, it has an enterobactin transport system that allows the organisms to use it as a xenosiderophore, even in chrysobactin-deficient mutants. A disruption of *cbs*H, an iron-regulated gene in *E. chrysanthemi* 3937 that has 45.6 % similarity to the *E. coli* ferric enterobactin esterase, results in disruption of the chrysobactin biosynthetic pathway. However the chrysobactin-deficient mutants are able to take up iron from ferric enterobactin when it is supplied exogenously, which promotes growth. Therefore we can conclude that a functional *cbs*H gene is needed for chrysobactin production but not ferric enterobactin uptake (Rauscher *et al.* 2002).

E. herbicola, previous name *Enterobacter agglomerans*, is a desferrioxamine E synthesiser and it is also thought to have an iron acquisition system that allows the organism to pirate iron from enterobactin removing 94.5% of the iron from the carrier; similarly coprogen can be pirated yielding 63.5 % of iron from the carrier whilst ferrichrome can also be used as an iron source with only 17.5 % of iron being removed in this case (Berner *et al.*, 1988).

Three strains of the fungus *Aspergillus* were tested for the ability to chelate iron from siderophores supplied in their cultivation media. *A. quadricinctus*, a ferrichrome synthesiser, could scavenge iron from ^{55}Fe labelled ferrichrome, ferricrocin and ferrirubin at similar transport rates. Nevertheless ferrichrysin-mediated iron uptake appeared to be significantly slower. *A. fumigatus*, a ferricrocin and ferrirhodin (a ferrirubin analogue) producer demonstrated similar rates of iron uptake for all the ferrichrome family. *A. melleus* showed the highest iron acquisition when ferrichrysin, its own siderophore, was provided. Ferricrocin, ferrichrome and ferrirubin were taken up by the isolate at significantly lower rates. It is worth mentioning that iron from coprogen and ferrioxamine B isolated from *Neurospora crassa* and *S. pilosus* respectively was not acquired from the strains, confirming that fungi posses a specific iron transport mechanism able to recognise only siderophores produced by its family (Wiebe & Winkelmann, 1975).

Some *Bacillus* strains are able to produce schizokonen or a catecholic compound, dihydroxybenzoate and itoic acid (dihydroxybenzolglycine). *Bacillus subtilis* SB164 only produces traces of catechol; but it is able to utilize at least three groups of hydroxamates: ferrichromes, ferrioxamines and shizokinen (Schneider & Hantke, 1993).

E. coli catechol-producing species are able to utilize the hydroxamates: ferrichrome, ferricrocin, ferrichrysin, coprogen and to a lesser degree ferrioxamines B, by possessing specific receptors in the outer membrane (Mullet *et al.*, 1984, Neilands, 1982 and Schneider & Hantle, 1993).

Heterobactins (A and B) produced by *R. erythropolis* IGTS8 act as xenosiderophores to both *E. coli* and *Agrobacterium flavescens* JG9. Although both compounds consist of catecholic moieties, *E. coli* takes up heterobactin A through a catechol-specific receptor (Cir) and heterobactin B through a hydroxamate one (FhuE). Both receptors facilitate the binding to enterobactin, a DHBA-serine conjugate (Carrano *et al.* 2001). Although the budding yeast *Saccharomyces cerevisiae* is unable to produce any siderophores, it contains a number of genes responsible for the expression of high-

affinity ferric reductases ameliorating iron acquisition from both hydroxamates and catechols (Howard, 1999). Ferrioxamine B, ferricrocin, rhodotorilic acid and ferrichrome are common iron sources for this organism (Howard, 1999 & Philpott *et al.*, 2002).

Candida albicans is the most abundant human fungal pathogen, causing infections to immunocompromised patients. It can also enter the blood steam and cause fatal systemic infections (Hu *et al.*, 2002). This strain possesses mechanisms attacking the erythrocytes and sequesters iron from haemoglobin. It also contains transporters (ferric and copper reductases) in order to acquire iron from ferrichrome and ferrichrome B, the process of which contributes widely to the strain's virulence (Howard, 1999).

Last but not least *Campylobacter jejuni* is a mammalian pathogen that survives and replicates in the gastrointestinal tract, causing numerous food-borne infections. Although it is yet unknown if the strain produces any siderophores, it is able to acquire iron from enterobactin. The organism lacks the genes responsible for the biosynthesis of the chelating agent; however it possesses the receptors for the xenosiderophore acquisition and transport. Mutants defective in enterobactin mediated iron acquisition are unable to colonise the gastrointestinal tract and cause gastroenteritis (Palyada *et al.*, 2004).

1. 6. 0. Siderophores and virulence

It is well established that iron is associated with the development of infectious pathogens. Without the ability to obtain iron, a pathogen will either die from the host's defence or by nutrient limitation. The virulence of many pathogens has significantly increased by experimentally adding iron to the host. These organisms include: enterobacteria, *Aeromonas*, *Clostridium*, *Corynebacterium*, *Listeria*, *Neisseria*, *Pasteurella*, *Staphylococcus*, *Yersinia*, *Vibrio*, *Mycobacterium* species and many more (Ratledge & Dover, 2000).

In contrast iron restriction induces exotoxin production in some pathogenic bacteria, which includes the toxins diphtheria, shiga and shiga-like in *Corynebacterium diphtheria*, *Shigella dysenteriae* and *Escherichia coli* respectively (Guster *et al.* 1993, Guster-Seeboth & Schupp, 1995).

Athough alcaligin (dixydroxamate) was originally isolated from the non-pathogen *Alkaigenes denitrificans*, it is also found in the respiratory pathogens: *Bordetella pertusis* and *B. bronchiseptica*. *Staphylococcus aureus* is a well-studied pathogen;

however the production of a novel siderophore, aureochelin (catechol), by the strain was only recently identified. The latter might imply that the absence of siderophore production by pathogens might reflect a lack of investigation rather than non-production per se (Ratledge & Dover, 2000).

Siderophore production is intimately related to the survival of *Brucella abortus*, an intracellular parasite able to multiply in macrophages, where the iron content is limited. Addition of the siderophore exogenously to murine macrophages injected with *B. abortus* increased the number of bacterial cells recovered compared to the untreated ones. However siderophore production is not involved in the survival of the strain in the phagosome. This might indicate that either iron concentration in the phagosome is high enough for the pathogen to proliferate or it has developed different mechanisms enabling it to acquire the element. Moreover *B. abortus* needs a very acidic phagosome during the first few hours of infection. At low pH iron is soluble and thus bioavailable and siderophores tend to be very unstable (Gonzalez Carrero *et al.* 2002).

Both chrysobactin and achromobactin act as a virulence factor in *Erwinia chrysanthemi* 3937 (Fraza *et al.* 2005). The siderophores are very important to this enterobacterium that causes soft rot pathogenesis on *Santpaulia ionantha* (African violet). The bacterium invades the intercellular spaces of the host's leaves and sometimes moves to other aerial parts of the plant. Ferric citrate is the major iron source in the plant tissues; however *E. chrysanthemi* 3937 is unable to utilize it. Therefore iron levels in the intercellular fluids are enough for the plant to grow but are not bioavailable to the bacterium. It must be noted that chrysobactin is only found in infected intercellular fluids (IIF) and not in healthy one (HIF). Although both fluids contain iron free ligands the IIF binds to iron 50 times more avidly than HIF, due to the siderophore production. Moreover the bacterium is unable to grow in HIF diluted with wash water. It seems that the media are lacking certain salts required for bacterial growth, such as iron chloride. However when the fluid (HIF) is concentrated 5-fold, which contains around 2μM iron, *E. chrysanthemi* 3937 does multiply and specific assays for strong iron ligands revealed that chrysobactin was present. Further studied with ^{59}Fe labelled ferritin incorporated in the plant cells revealed a substantial decrease of plant ferritin iron and consequently a substantial increase of bacteria ferritin iron. Therefore although chrysobactin is essential for bacterial growth it has a deleterious effect on the host as it starves it for iron (Neema et *al.* 1993).

Vibrio vulnificus is a marine bacterium responsible for causing primary septicaemia and wound infection. In humans the former is contracted by eating raw oysters or shellfish, whereas the latter is caused by exposure of wounds to seawater. Septicaemia is also associated with iron overload. *V. vulnificus* produces a catechol, vulnibactin, which is strongly associated to the virulence of the bacterium. A mutant unable to produce the siderophore or to acquire iron from transferrin presented significantly reduced virulence. The mutant demonstrated 50 % lethal dose (LD_{50}) of 10^7 on mice after 20 hours whereas the wild type had a much lower LD_{50}, which was of 6.3×10^4. LD_{50} is the dose of the compound needed to kill 50 % of the population. Moreover avirulent strains failed to produce the chelating agent, which explains the relationship between siderophore production and pathogenisity of the bacterium (Litwin *et al.* 1996).

Vibrio salmonicida excretes a di-hydroxamate compound, bisucaberin, and also causes septicaemia, "cold water vibriosis", in Atlantic salmon. There is evidence that the siderophore production is not only intimately related to the organism's virulence but is temperature controlled as well (Winkelmann, 2002). Although the organism is still growing at temperatures up to 15^0 C, siderophore production is repressed. Outbreaks of the infection are reported mostly at 4-8^0 C and rarely above 10^0 C. These data are important for the vaccine manufacturing business. It has been proposed that although poikilothermic organisms (fish) do not show any negative reactions against an increase in temperature, i.e "fever", the *Vibrio* strain is indeed affected and thus loses its ability to produce the siderophore (Coiquhoun & Sorum, 2001).

Another strain of *Vibrio*, *Vibrio anguillarum* 775, produces anguibactin (catechol), a cystein-conjugate, which is an enterobactin-related compound. The organism restricts the cultivation of salmon globally and its virulence is related to a 65Kb plasmid, pJM1, which carries iron acquiring specific genes responsible for the siderophore production (Neilands, 1982 and Pybus & Loutit, 1994).

Additionally, *Vibrio cholerae* excretes a catecholic compound, vibriobactin. Although the latter is associated with pathogenesis, the organism also has a second mechanism of acquiring iron, which involves the lysis of the host's heme and the consequent release of iron. Both mechanisms appear to be important and disruption of genes responsible for either route reduces the virulence of the isolate (Ratledge & Dover, 2000).

The virulence of at least 58 *E. coli* strains that cause urinary tract infections, such as pyelonephritis, cystitis or urosepsis, and the relationship with aerobactin gene location

has been studied. There is evidence that chromosomal-associated aerobactin is present in patients without any urological history. However plasmid-associated aerobactin is present in patients with problematic health and also display resistant to multiple antimicrobials. It is believed that the aerobactin, microbial-resistant plasmids evolved in the compromised patients through previous conduct with antimicrobial therapies (Johnson *et al.* 1988).

Enterobacteria are well known to produce catecholic compounds, which are directly related to their virulence. Certain species are nosocomal pathogens, causing opportunistic infections in hospitals. Genes responsible for the siderophore production and uptake in some of these organisms are located on pathogenicity islands (SHI-2, SPI-1, HPI). These islands can contribute to the formation of new pathotypes as they can easily acquire new virulence related functions through gene exchange. SHI-2 of *Shigella flexneri* carries genes encoding aerobactin production. SPI-1 in *Salmonella typhimurium* contains genes for iron transport. *Yersinia enterocolitica* and *Y. pseudotubrculosis* possess a high pathogenicity island (HPI) responsible for the production of a chromosomal-related catechol, yersiniabactin, which is a major virulent factor (Carniel *et al.* 1996).

Yersiniabactin is very similar to pyochelin produced by *Pseudomonas aeruginosa* and *Burkholderia cepacia*, which have an extra thiazoline ring. Both microorganisms are associated with severe respiratory infections in cystic fibrosis patients and also produce pyoverdin (or pseudobactin), ornibactin and capabactin. *P. aeruginosa* mutants deficient in pyoverdin production but not pyochelin possess a significant decrease in pathogenesis, suggesting that pyochelin is the main contributing virulence factor. Furthermore, ornibactin deficient mutants appear to be less virulent than the wild type in rats and mice (Ratledge & Dover, 2000).

Clinical isolates of *Enterobacter* and *Citrobacter* species, which are known to produce enterobactin, aerobactin or in less cases a different type of hydroxamate, have been examined for their ability to produce yersiniabactin. The results revealed that *E. cloaceae*, *E. aerogenes* and *C. koseri* are able to produce this siderophore, which confirms the presence of a functional HPI. The additional properties of these clinical isolates raise immediate concern about their spreading in hospitalised environments (Mokracka *et al.* 2004).

Clinical material is often used as a source of pathogenic actinomycetes that produce antimicrobial compounds used for anti-tumour drugs or immunosuppressives. In 1997 an actinomycete classified as *Actinomadure madurae* was isolated from a bronchitis

patient at Chiba University Hospital, Japan. The organism was reported to produce novel compounds, named madurastatins (A1, A2, A3, B1 and B2), which is believed to contribute to the pathogen's virulence. A1 and B1 present antimicrobial activity against *Micrococcus luteus* and A1 is listed as a strong chelating agent that contains two hydroxamic moieties. Although madurastatin A1 enhances the isolate's pathogenisity and the resistance to antibiotics (bronchitis' patients), the compound can be used as an efficient antimicrobial against *Micrococcus luteus* (Harada *et al.* 2004).

Mycobacteria genus includes species that are major pathogens, which infect both humans and animals. *M. tuberculosis* and *M. leprae* are causative agents of tuberculosis and leprosy respectively. Moreover *M. avium*, a causative agent of avian tuberculosis causes concern, as it is extremely difficult to eradicate the infection from humans, is very common to immunocompromised/HIV patients and a major mortality factor to them (Sharman *et al.*, 1995). Animal model studies have shown that when mice were fed with iron-rich diets, they rapidly developed the disease. Moreover in cultured human macrophages the rate of the infection elevated with the increasing iron concentration (De Voss *et al.*, 1999).

Additionally pulmonary tuberculosis patients are often anaemic, suggesting lack of bioavailable iron to the host. In an attempt to treat what was thought iron deficiency in infected Somalian patients, iron tablets was discovered to promote the strain's virulence and enhance the risk of death (De Voss *et al.*, 1999 & De Voss *et al.*, 2000).

M. tuberculosis, *M. leprae* and *M. avium* all produce two types of siderophores: the extracellular carboxymycobactins and the intracellular, lipid-soluble, mycobactins. The extracellular siderophores produced by non-pathogenic mycobacteria are named 'exochelins' so they can be differentiated from the ones produced by pathogenic species. Exochelin MS is produced by *M. smegmatis* and it is a trihydroxamate containing threonine and alanine; whereas exochelin MN produced by *M. neoaurum* is an alanine-ornithine conjugate (Sharman *et al.*, 1995).

Mycobactins serve as short-term iron storage system, which is readily available, while the carboxymycobactins pass into the cytoplasm through the thick lipophilic cell envelope (De Voss *et al.*, 2000).

Carboxymycobactins are water soluble, modified mycobactins and can solubilise iron from various sources including tranferrins (Tfs). The quantity of the siderophore produced by *M. tuberculosis* and *M. bovis* (causative agent of tuberculosis in cattle) increases with the virulence of the strains, providing evidence that

carboxymycobactins are closely related to pathogenicity. There is evidence that both intracellular and extracellular siderophores are not needed at the same time as in mycobactin defect strains of *M. paratubercolosis* carboxymycobactins replaces the requirement for mycobactin (Ratlege & Dover, 2000).

1. 7. 0. Production of iron-binding compounds as a response to iron deficiency in Actinomycetes

1. 7. 1. Mycobacteria

In April 1993 tuberculosis was declared as a global health emergency, presenting the first such urgent designation in the history of the World Health Organisation (WHO). Therefore in order to develop a new, more effective tuberculosis control, it is critical to investigate *M. tuberculosis* at genome level and compare it with saprophytic, non-pathogenic related ones, such as *S. coelicolor*' (Bardarov *et al.*, 1997).

Mycobactins are synthesised by a group of ten proteins. The non-ribosomal peptide synthases (NRPSs) include two polyketide synthetases (MbtC and MbtD), three peptide synthetases MbtB, MbtE and MbtF) and one isochorismate synthase (MbtI), which provides salicylic acid (Figure 11). MbtB is thought to activate serine and condense it with salicylate, which has already been activated by MbtA, an acyl adenylate, and finally produce a cyclic hydroxyphenyloxazoline. MbtE and MbtF activate and condense two lysine moieties and MbtC and MbtD are responsible for the β-hydroxybutyrate. Moreover MbtG is an ornithine and lysine oxygenase, which is required for the formation of ε-*N*-hydroxylysines. These seven proteins are mainly involved in the biosynthesis of the mycobactin core. There are two more related proteins, MbtH and MbtJ, the biochemical role of which is not yet clear (De Voss *et al.*, 1999).

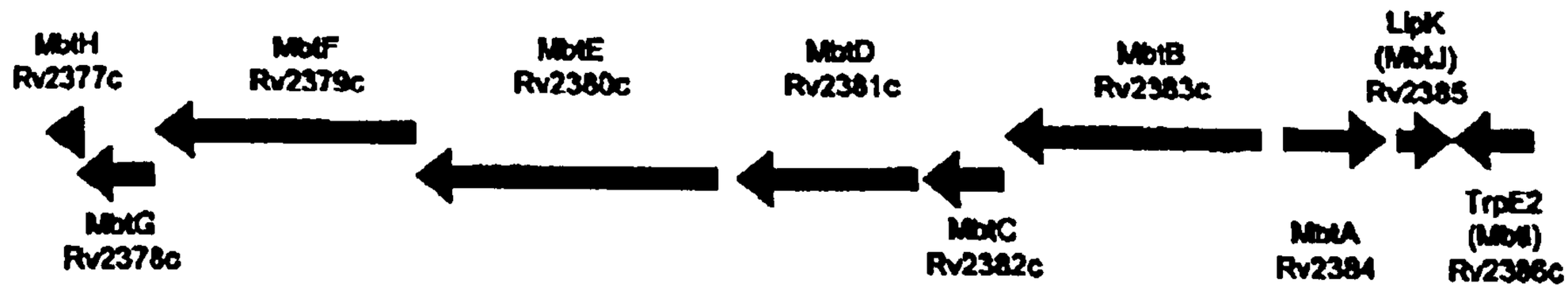


Figure 11: Genome of *M. tuberculosis* containing the mycobactin biosynthetic enzymes. Redrawn from De-Voss *et al.*, 1999.

Disruption of MbtB in *M. tuberculosis* H37Rv has resulted in a mutant unable to synthesise the siderophore and survive in macrophages, turning this protein into a

target for new antibiotics against tuberculosis-related infections. Moreover MbtB is 35 % identical to Ybt from *Yersinia pestis*, responsible for the high pathogenicity island (HP2) regulated yersiniabactin core formation (De Voss *et al.*, 2000). Exochelin biosynthetic pathway in *M. smegmatis* has been extensively studied (Fiss *et al.*, 1994 and Yu *et al.*, 1998) and a NRPS cluster associated with the siderophore production (MS) has been identified. Three genes have been elucidated to be crucial, *fxbA*, *fxbB* and *fxbC*. Genes: *fxbA* encode for a transferase and *fxbB* and *fxbC* are associated with synthetases. Three open reading frames (ORFs) directly upstream to *fxbA* are similar to the enterobactin iron transporters of *E. coli* and therefore these ORFs have been suggested to contribute to the isolate's iron transport system (Rodriquez & Smith, 2003).

1. 7. 2. *S. coelicolor*

The siderophores coelichelin is a hydroxamate tetrapeptide ($_D$-hfOrn-$_D$-alloThr-$_L$-hOrn-$_D$-hfOrn), whose biosynthetic pathway (NRPS regulated) involves 15 genes (*cchA-cchO*), containing the *cch* (coelichelin) cluster (Figure 12). *CchA* and *cchB* encode for $_L$-5-hydroxyornithine 5-formyl transferase and a $_L$-ornithine 5-monooxygenase, enzymes responsible for the concentration of $_L$-ornithine to $_L$-5-hydroxy-5-formylornithine and $_L$-5-hydroxyornithine, which represent the first and third residue of coelichelin structure. Genes: *cchC*, *cchD*, *cchE*, *cchF*, *cchG* and *cchI* code siderophore-binding lipoproteins or siderophore transporters (Challis & Ravel, 2000). There is a strong resemblance between the cchJ and the enterobactin esterase, which is not required for the enterobactin production. In order to investigate further the function of this protein, the *cchJ* of *S. coelicolor* M147 was replaced creating a mutant called *S. coelicolor* W6. Surprising strain W6 was unable to produce coelichelin, suggesting that *cchJ* acts as a thioesterase, whose hydrolytic function releases the fully assembled tetrapeptide from the NRPs (Lautru *et al.*, 2005).

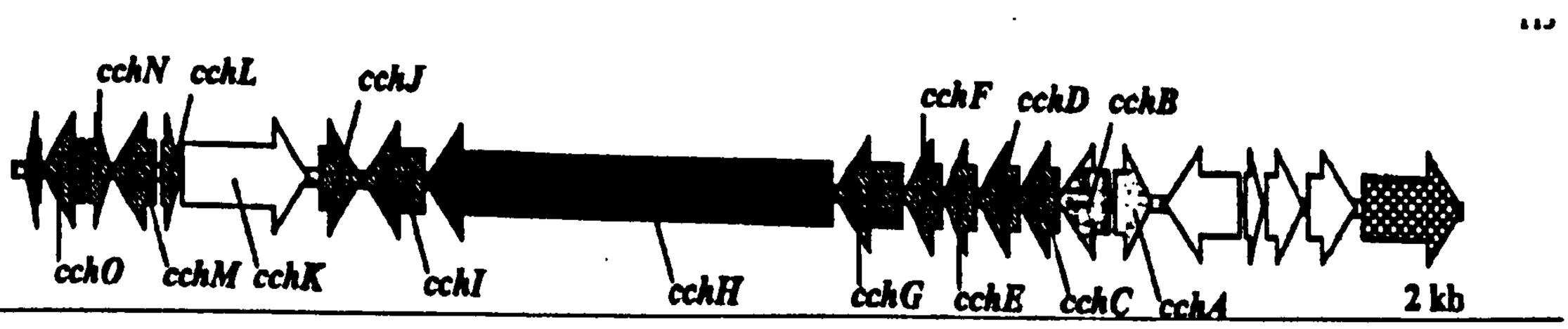


Figure 12: Organisation of the coelichelin biosynthetic gene cluster. Redrawn from: Challis & Ravel, 2000.

Investigation of the recently sequenced genome of *S. coelicolor* revealed an operon containing four genes *desA-D* associated with desferrioxamines G1 and E biosynthesis (Figure 13), whose pathway is non-ribosomal peptide synthetase (NRPS) independent (NIS). Genes *desA* and *desB* represent decarboxylases and monooxygenses respectively that catalyse the first two steps of hydroxamate production. The protein product of *desC* acts as acyl CoA transferase and cyclization is catalysed by *desD*. The IdeR binding site is located directly upstream of *desA*, indicating that the system is iron-dependent.

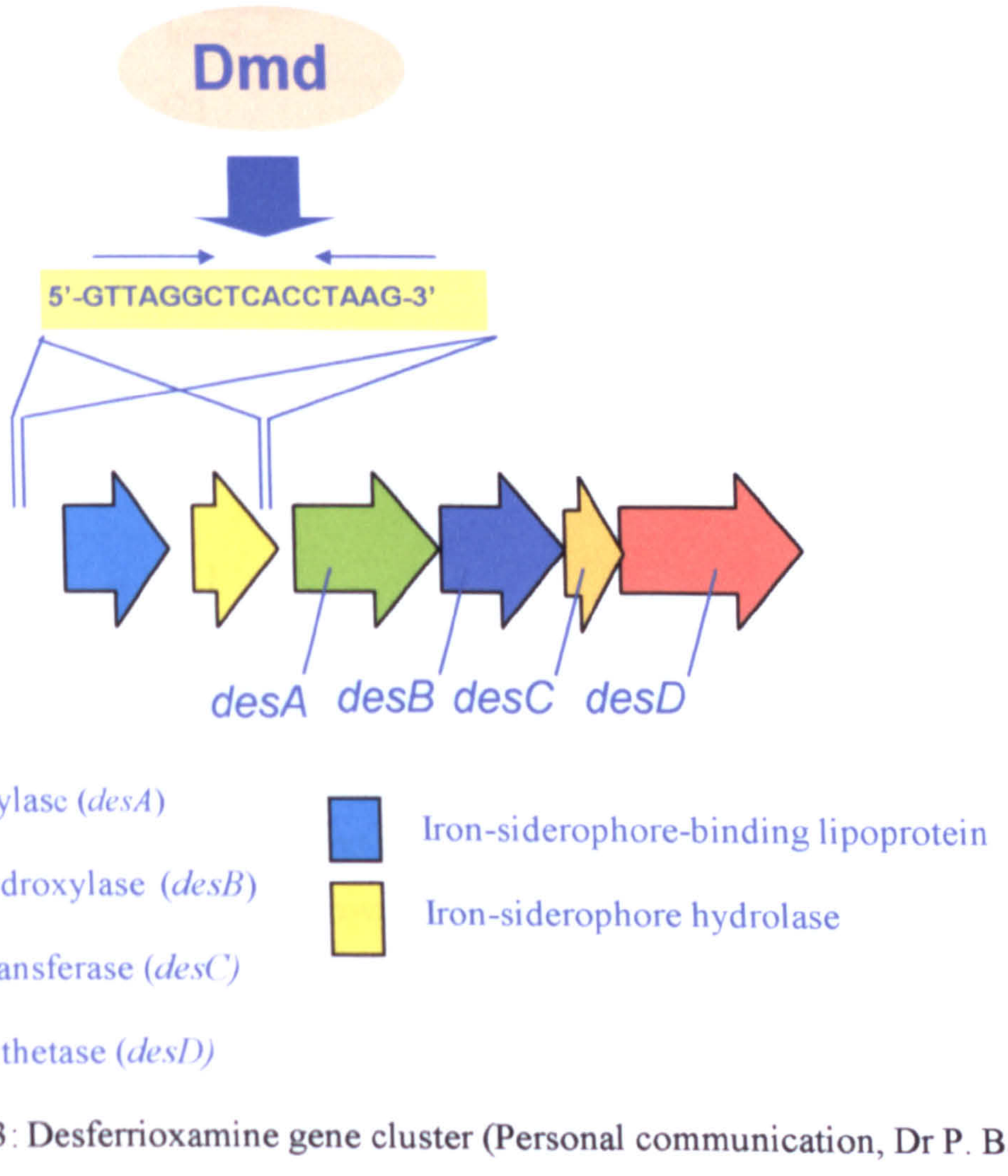


Figure 13: Desferrioxamine gene cluster (Personal communication, Dr P. Barona-Gomez).

In order to investigate further the function of *desD* in *S. coelicolor* M145 a mutant W2, whose *desD* gene had been replaced, was constructed. Under iron-deficient media the Isolate M145 was found to produce both desferrioxamine G1 and E (main).

Amending the media with 36 μM iron completely repressed siderophore production; nevertheless strain W2 was unable to produce any hydroxamates, indicating that *desD* plays a crucial role in the chelate agent's synthesis (Figure 14). Deletion of *desD* was complimented with a derivative of the cosmid SCC105, which contained the *des* operon, restoring hydroxamate production (Barona-Gomez *et al*., 2004). Moreover only when double *des* and *cch* mutants were constructed did growth under iron-deficiency become impaired. Unpredictably growth of *S. coelicolor* W13, defective for the entire *des* and *cch* gene clusters was restored by adding several hydroxamates, suggesting that a third transport system must be present in *S. coelicolor*, supporting the contingency theory (personal communication F Barona-Gomez, unpublished data).

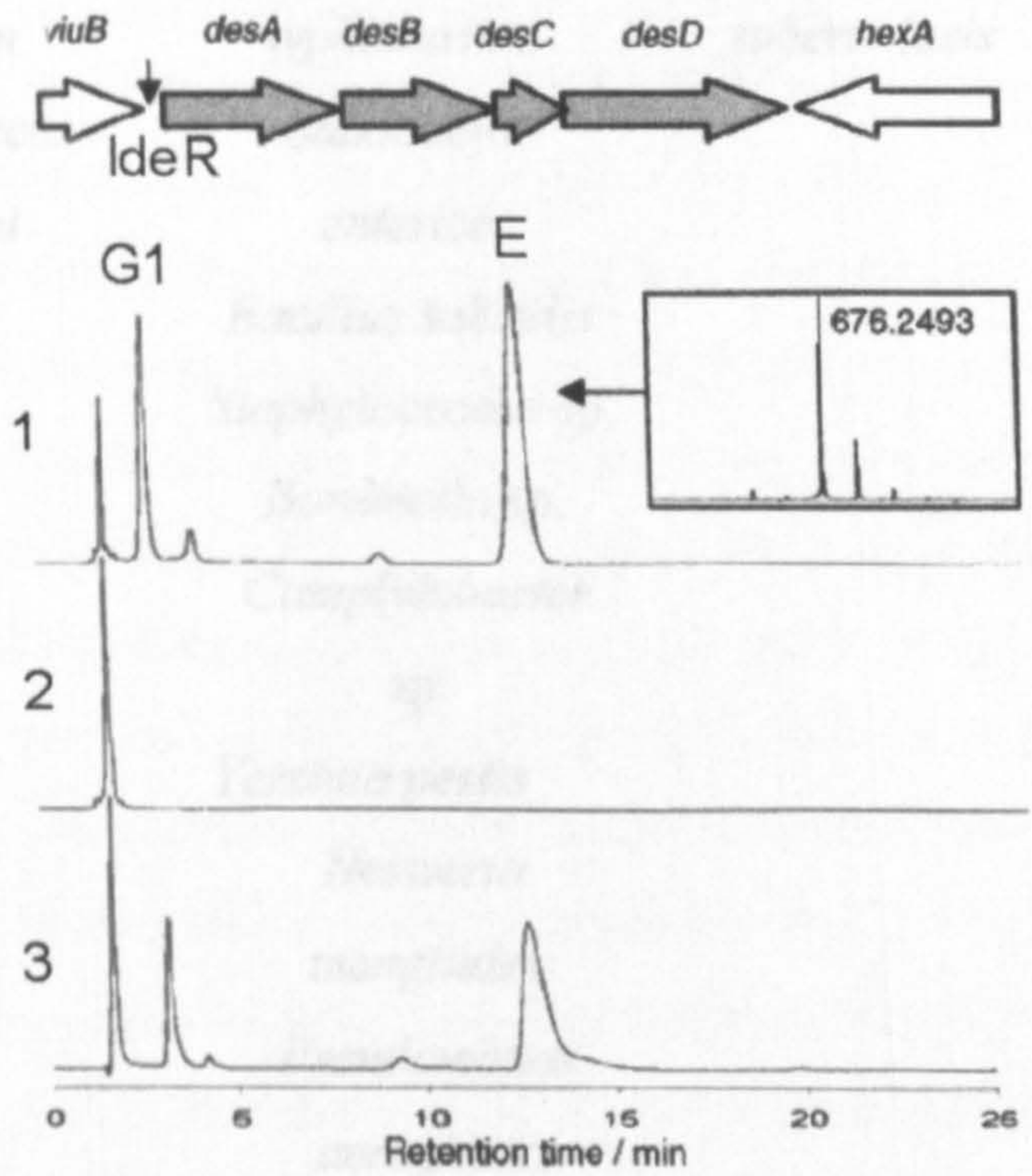


Figure 14: Organization of the *des* operon in *S. coelicolor* M145 (top). HPLC analysis of desferrioxamines G1 and E production by *S. coelicolor* M145 (1), W2 (2) and W2 +SCC105 (3). Redrawn from Barona-Gomez *et al*., 2004).

1. 8. 0. Iron-dependent regulation in bacteria

The response to different concentrations of iron is controlled in bacteria by iron-dependent regulatory proteins. Although there is much confusion in terms of terminology used in the literature, in general there are two distinct groups: the Fur and the Fur-like homologs: DtxR/DmdR families (Table 5).

Iron dependent regulatory proteins (homologs)				
	DtxR	Fur	IdeR	DmdR
ORGANISMS	*Corynebacterium diphteriae* *Treponema pallidum* *Streptococcus gordonii*	*Escherichia coli* *Salmonella typhimurium* *Salmonella enterica* *Bacillus substilis* *Staphylococcus* sp. *Bordetella* sp. *Camplylobacter* sp. *Yersinia pestis* *Neisseria mengitides* *Pseudomonas aeruginisa* *Legionella pneumophila* *Helicobacter pylori* *Vibrio* sp. *Erwinia chrysanthemi* *Bradyrhizobium japonicum*	*Mycobacterium smegmatis* *Mycobacterium tuberculosis*	*Streptomyces coelicolor* *Streptomyces lividans*

Table 5: Description of the iron regulatory proteins present in bacteria.

1. 8. 1 Fur proteins

Fur (ferric uptake regulator) proteins, encoded by *fur* genes, are transcriptional repressors that when attached to the co-repressor Fe^{2+} bind to specific sequences in iron regulated promoters (Fur boxes) inhibiting downstream transcription of genes associated with siderophore production. The target sequence of *E. coli*'s Fur box is: 5' GATAATGATAATCATTATC3' (Escolar *et al.*, 1999).

Fur identification is commonly performed by the Fur titration assay (FURTA), which has been extensively reviewed in the literature (Escolar *et al.*, 1999, Stojiljkovic *et al.*, 1994 and Tsolis *et al.*, 1995). Moreover searching for *fur* mutants in organisms other than *E. coli* might reveal important information about the iron-responsive promoters. A very effective method is screening for manganese-resistant colonies. The reason behind this is that high concentrations of Mn^{2+} mimics Fe^{2+} and therefore causes a lethal repression of the iron uptake system. Cells that lack Fur escape and can be picked off a plate and analysed further (Escolar *et al.*, 1999)

The importance of these regulators was demonstrated in 1981 with *E. coli* and *Salmonella fur* defective mutants; although they appear to be competent in iron transport, they had lost their ability to control the biosynthesis of iron chelating agents under any iron concentrations (Bagg & Neilands, 1985). That was the first example of metal dependent repression, which subsequently led to the mapping, cloning and sequencing of the gene and additionally to the purification of its protein product.

E. coli's iron regulation mechanism is the most studied and includes at least five different siderophore acquisition systems, which are encoded by more than 20 genes distributed in various operons across the chromosome (Stojiljkovic *et al.*, 1994).

In *E. coli* Fur can control the transcription or *purR*, a repressor that regulates the expression of eight purine nucleotide synthesis operons. In the same organism methionine biosynthesis is regulated by two proteins, *metJ* (repressor) and *metR* (activator), which can be up regulated or down regulated by the Fur depending on the cells demands (Stojiljkovic *et al.*, 1994).

Moreover Fur can positively regulate the transcription of certain molecules. A paradigm of this mechanism is *E. coli*, where Fur represses the synthesis of small RNA molecules that stop translation of mRNAs for bacterioferritin and ferritin. Fur regulates a number of processes, including oxidative stress response, chemotaxis, acid tolerance, toxin biosynthesis and virulence (Escolar *et al.*, 1999 and Rodriguez & Smith, 2003).

Homologs of the *E. coli fur* gene has been identified in various gram-negative bacteria, including *Bordetella* sp., *Campylobacter* sp., *Yersinia pestis*, *Neisseria meningitides*, *Pseudomonas aeruginisa*, *Legionella pneumophila*, *Bradyrhizobium japonicum*, *Helicobacter pylori*, *Acinetobacter baumanni*, *Erwinia chrysanthemi*, *Vibrio vulnificus*, *Vibrio cholerae* (Escolar *et al.*, 1999, Hamza *et al.*, 1999 and Watnick *et al.*, 1997).

Fur-like proteins have also been described in gram-positive bacteria, such as *Bacillus subtilis* and *Staphylococcus* sp., and even in some cyanobacteria. Most of these proteins can complement an *E. coli fur* mutant suggesting that the mechanism that control iron regulation must be shared by many bacteria (Escolar *et al.*, 1999)

V. cholerae's *fur* gene was over expressed in *E. coli* and purified to 95 % homogeneity. It was reported that the number of Fur molecules per cell during exponential phase was 2500, reaching its maximum, 7500, during stationary phase (Table 6). To our knowledge this is the highest *fur* expression. In *V. cholerae* a large number of genes are regulated by Fur but only 22 proteins are known to be repressed in the presence of iron (Watnick *et al.*, 1997).

OD_{600}	Fur (ng/ml)/OD_{600} in culture	Number of Fur molecules/bacterial cells
0.671	188	2618
1.140	308	5909
2.050	529	7414

Table 6: Measurements of the intracellular abundance of Fur in *V. cholerae* 0395 cultures at various stages of growth. Redrawn from Watnick *et al.*, 1997.

Furthermore it was demonstrated that the transcription of 647 genes was affected by the addition or iron in iron-starved *C. jejuni* cells. The genes were responsible for various functions including energy metabolism, oxidative stress and iron acquisition. Microarray studies revealed that in a Fur *C. jejuni* mutant the expression of 53 genes were Fur regulated. It was also demonstrated that the promoter of the iron repressed and Fur regulated genes possessed a Fur binding site. Interestingly the mutant's virulence was significantly affected demonstrating the importance of Fur in the pathogenesis of the organism (Palyada *et al.*, 2004).

So far Fur appears to be exclusively a repressor. Its function as a transcriptional activator has never been proven. The direct relation ship between the Fur protein and

the promoter of iron- activated genes has never been described. Therefore it can be concluded that Fur remains a repressor and its positive effect on specific promoters is thought to be a result of an indirect rather that direct interaction (Escolar *et al.*, 1999).

1. 8. 2 DtxR proteins

DtxR and Fur, although structurally dissimilar (only 20% identity at the amino acid level), share the same function (homologues). DtxR was originally identified in *C. diphteriae* and regulates iron uptake in gram-positive bacteria with high GC content, such as actinomycetes including streptomycetes and mycobacteria species (Hantke, 2001). They are well known to control genes encoding iron transportation, a heme oxygenase, virulence and protection against oxidative stress. They also regulate metal binding, acting as a transcription repressor. TroR from *Treponema pallidum*, ScaR from *Streptococcus gordonii* and MntR from *B. substilis* and *E. coli* are all DtxR-like proteins regulating Mn^{2+} acquisition and transport mechanisms (Rodriguez & Smith, 2003). DtxR is sometimes referred as DmdR (divalent metal-dependent regulator) due to its ability to bind bivalent metals (Flores & Martin, 2004 and Hantke, 2001).

1. 9. 0. Co-regulation of iron metabolism and oxidative stress:

Regulation of iron metabolism is intimately linked with protection against oxidative stress caused by the intracellular accumulation of abnormally high levels of oxygen radicals. Iron deficiency stimulates oxidative stress by reducing the activity of iron-containing enzymes, such as catalases; whereas iron overload can lead to generation of high levels of oxygen radicals causing irreversible DNA, protein and lipid damage (Palyada *et al.*, 2004).

Two of the most important bacterial oxidative stress related genes are the catalase peroxidase and the alkyl hydroperoxide reductase encoded by *KatG* and *AhpC* respectively. The expression of these genes is regulated by *OxyR* in response to increasing iron concentrations in the environment. Both *KatG* and *AhpC* contain Fur binding consensus sequences, which identifies Fur as the regulator. This mechanism occurs in most bacteria, including *E. coli*, *S. typhimurium*, *M. leprae* (Van Vliet *et al.*, 1999).

C. jejuni is the first gram-negative bacterium where non *OxyR* regulations has been described. Although iron repression is normally carried out by Fur, *C. jejuni Fur* mutant still demonstrates iron-repressed genes, which implies a different regulation mechanism. A Fur homologue, *PerR* (peroxide stress regulator), was identified in the

organism's genome and a *C. jejuni PerR* mutant shows derepression of both *KatG* and *AhpC* in much higher levels that those obtained under iron starvation. A double *Fur*, *PerR C. jejuni* mutant reveals repression of all known iron-repressed genes, confirming that both Fur homologues are coregulating oxidative stress. It is worth mentioning that *PerR* shares the same function with *OxyR* but they are non-homologous, which means they do not share a high sequence identity (Van Vliet *at al.*, 1999).

In Mycobacteria FurA proteins regulate the genes induced by oxidative stress, such as *katG*, which is located directly downstream of the *furA* (Rodriguez & Smith, 2003). IdeR mutants of *M. smegmatis* and *M. tuberculosis* demonstrated elevated sensitivity to oxidative stress indicating that the iron regulator is required for the full expression of *katG*. FurA proteins cluster with the FurS from *S. reticuli*, which also regulates the catalase peroxidase, *cpeB* (De Voss *et al.*, 1999).

The relationship between oxidative stress and iron regulation was also demonstrated in the Actinomycete, *S. coelicolor*, where two genes *furA* and *catC* were isolated and found to regulate iron uptake and catalase peroxidase respectively. *FurA* is located upstream to the *catC* and they appeared to be co-transcribed. The role of FurA as a negative regulator of *FurA-catC* was demonstrated by introduction of this operon into *S. lividans*, where *catC* expression was inhibited. Moreover addition of Ni^{2+}, Mn^{2+}, Zn^{2+} and Fe^{2+} significantly decreased the production of *catC* demonstrating that FurA protein is probably a bivalent metal repressor (Hahn *et al.*, 2000).

1. 9. 1. Fur related metalloregulatory proteins

The regulatory mechanism described for Fur has applications on other metal repressors too. Metalloregulatory proteins regulate the intracellular levels of specific metal ions and initiate transcriptional response (Gaballa & Helmann, 1998).

A good example is *B. subtilis'* genome, which contains three open reading frames (ORFs) all encoding Fur homologues. One is responsible for iron uptake regulation (Fur), one for *OxyR* (previously mentioned at coregulation of iron metabolism and oxidative stress section) and the third functions as zinc uptake regulator (Zur). All these metalloregulators are significantly related to Fur proteins from both gram-positive and negative bacteria (Van Vliet *et al.*, 1999).

Like iron, zinc can be an absolute essential element or toxic to the cell. Moreover Fur and Zur have significant sequence identity. Similarly *Zur* regulates zinc acquisition and transport to the cell repressing the mechanism in the presence of high elemental

(zinc) concentrations. The exact repression system is not well described but there is some evidence that there are Zur binding sequences specific for the promoters of genes involved in zinc sequestration.

In *B. subtilis* the process involves at least two promoters: the *ycdH* containing, which encodes a high affinity zinc transport system and the *yciC* containing with still an unknown function. The total number of genes under the Zur protein control is yet to be elucidated. Interestingly Zur 'box' demonstrates a 12 out of 19 nucleotides similarity to the Fur box consensus sequence (Gaballa & Helmann, 1998 and Van Vliet *et al.*, 1999).

Multiple Fur homologues have been previously reported for various gram-positive bacteria, including *E. coli*, *Streptococcus pneumoniae*, *Listeria monocytogenes* and *Staphylococcus aureus*, whose second Fur homologue has also been described as a Zur (Bsat *et al.*, 1998, Escolar *et al.*, 1999, Gaballa & Helmann, 1998 and Van Vliet *et al.*, 1999).

Zinc uptake regulators have also been identified in gram-negative microorganisms, including *Pseudomonas aeruginosa*, *Salmonella enterica*, *Haemophilus influenzae* and *Synechocystis* strain PCC 6803 (Campoy *et al.*, 2002 & Gaballa and Helmann, 1998).

There is no doubt that similarities exist between the regulation of iron and zinc uptake systems; however there is little and in many cases contradictory information about the importance of zinc in bacterial virulence. Most of the data available refer to extracellular pathogens. It has been demonstrated that *Haemophilus influenza* and *P. aeruginosa zur* mutants are less virulent than the wild type. However the virulence of a *S. aureus zur* mutant is not affected at all. The only study regarding the role of zinc in intracellular pathogens to our knowledge is with *Salmonella enterica*, where the *zur* defective strain demonstrated an increase in the number of cells required to achieve LD_{50} in mice (Campoy *et al.*, 2002).

Moreover the literature is confused in terms of nomenclature as some proteins previously described as Fur are definitely more similar to Zur that Fur. These proteins have to be reclassified as zinc uptake regulators. On the other hand many Zur proteins have been characterised as Fur adding even more complications. For example in 1999 Fur has been characterised as a Zn-metalloprotein, containing 2.1 mol of zinc (II) / mol of Fur monomer (Althaus *et al.*, 1998). There are even indications that Fur proteins contain two binding sites, one for Fe^{2+} and one for Zn^{2+} (Hantke, 2001).

Last but not least similar metalloregulation proteins have been identified for Hg^{2+}, Cd^{2+}, As^{2+}, Co^{2+}, Ni^{2+}, Mn^{2+} (Grass *et al.*, 2004, Hantke, 2001, Kim *et* al., 2001, Mireles *et* al., 2005, Pena *et al.*, 1999, Roberts *et al.*, 2001, Saito *et al.*, 2002, Saito *et* al, 2004, Saito *et* al, 2005 & Satish *et al.*, 2004) ...

1. 9. 2. Mycobacteria:

Regulation of gene expression in the presence of iron has been extensively studied in mycobacteria. In *M. tuberculosis*, IdeR, a functional homologue of DtxR, regulates iron metabolism (Cronje *et al.*, 2005). When intracellular iron levels increase IdeR combines with Fe^{2+} and bind to a 19bp inverted repeat consensus sequence of the iron box (TTAGGTTAGGCTAACCTAA) at the promoters of *mbtA-J* and iron storage genes (*bfrA-B*) transcriptionally repressing the former and positively regulating the latter. In these conditions IdeR represses siderophore production and increases iron storage by activating the transcription of *bfrA and bfrB* encoding bacterioferritin and ferritin. Under iron starvation IdeR no longer binds to the fur box and the corresponding genes become derepressed (Figure 15) [Rodriguez & Smith, 2003]. IdeR also binds to other bivalent metals, including Mn, Zn, Co, Ni and Mg. The requirement of this regulator in the presence of elevated iron levels was demonstrated in *M. smegmatis* by successfully inactivating the IdeR, resulting in a mutant able to produce siderophores under high and low iron concentrations (De Voss *et al.*, 1999). Nevertheless IdeR is essential to *M. tuberculosis* and it cannot be disrupted unless a second copy of the regulator is present. Disruption of IdeR also prevents the expression of *bfrA and bfrB* and consequently the iron storage (Rodriguez & Smith, 2003).

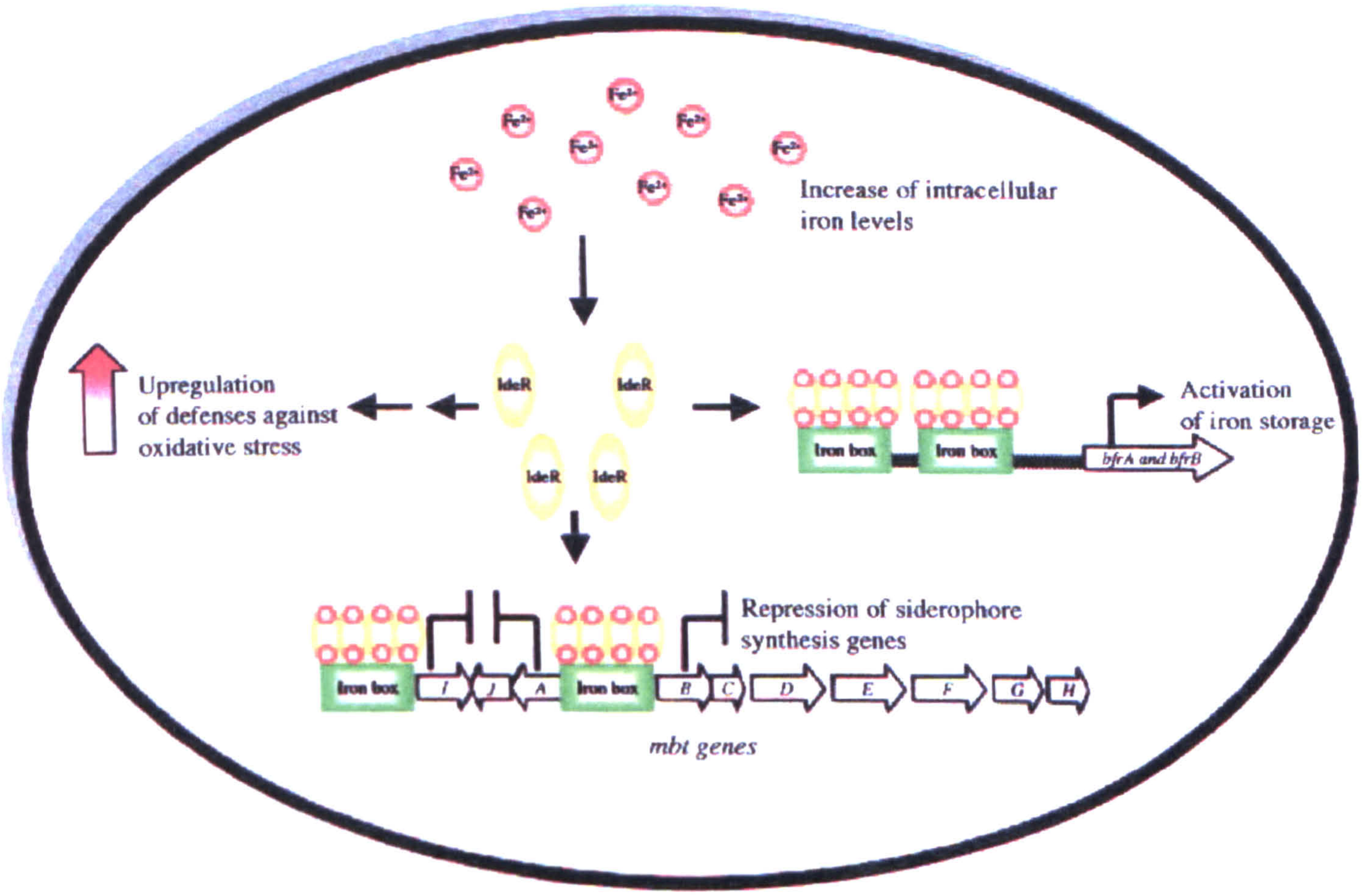


Figure 15: Iron-dependent regulatory function of IdeR in *Mycobacterium tuberculosis*. Redrawn from Rodriguez & Smith, 2003.

1. 9. 3. *Streptomyces*:

Recently it has been discovered that *S. coelicolor* contains at least two DtxR-like proteins, DmdR1 and DmdR2, and up to ten iron boxes. Disruption of the *dmdR1* gene results in derepression of four proteins and repression of five other, whereas deletion of *dmdR2* has no effect on the protein profile of the strain. Although their role as iron regulators has been established the regulatory mechanism is still not clear (Flores & Martin, 2004).

The iron-regulated promoter, *desA*, involved in desferrioxamine B biosynthesis of *S. pilosus* has been characterised and a repressor binding site (iron box) similar to the DtxR of *C. diphtheria* has been identified. (Gunter *et al*, 1993). It has also been reported that purified DmdR1 and DmdR2 from *S. coelicolor* bind to the *desA* with DmdR2 demonstrating higher affinity for the operon compared with DmdR1 (Flores & Martin, 2004 and Flores *et al*., 2003). Hence it has been suggested that the *desA*

promoter can be used for inducible or repressible expression in different actinomycetes (Flores *et al.*, 2003). Furthermore *S. lividans* contains a DtxR homologue, which encodes an iron transcriptional regulator (Gunter-Seeboth & Schupp, 1995).

1. 10. 0. Siderophore applications

Siderophores possess important pharmaceutical activity and in addition have potential environmental applications in bioleaching.

1. 10. 1. Clinical applications

In an attempt to discover more efficient anti-tuberculosis drugs the concept of chelation therapy was introduced back in the 1960s, where mycobactin was investigated as part of a commercial program. Unfortunately it was soon abandoned, as at this time it was considered, due to the receding cases of Tuberculosis, that the drug would not be profitable. Ironically one of the first such drugs used to successfully treat TB, *p*-aminosalicylic acid (PAS), is well known to inhibit iron metabolism (Ratledge & Dover, 2000).

Furthermore excess iron is implicated in encouraging nosocomical infections by increasing virulence of opportunistic pathogens (Meyer, 2006). In addition there is convincing evidence that removal of iron by chelation or phlebotomy improves the treatment of patients suffering from clinical manifestations such as, iron overload, thalassemia, Tuberculosis, malaria, hepatitis C and AIDS (Cronje *et al.*, 2005 & Tam *et al.*, 2003).

The use of iron chelators as anti-tumour therapies has also been explored.

Siderophores are proven to be useful anti tumour agents by depleting iron and causing oxidative stress to the tumour. Although it has already been demonstrated that desferrioxamines can reduce tumour growth, the activity is still modest requiring further investigation. Additionally apart from the application of siderophores as single anti tumour agents, they can also be used synergistically with traditional anti-cancer therapies, already established, in order to further improve treatment. The latter still remains an unexplored field (Buss *et al.*, 2003).

Desferrioxamine B produced by *S. pilosus* (trade name Desferal) is marketed as the mesylate salt and it is used as a drug for iron chelation subsequent to iron intoxication; a side effect of therapy for thalassemia, idiopathic hemochromatosis and

transfusional hemosiderosis. The drug has limited intracellular access and is injected directly to the patients' blood stream. Nevertheless there is an increasing need for an oral replacement (Cramer, 1984 & Neilands, 1995). However more recent studies indicate that administration of desferrioxamine to patients suffering from thalassemia increases the therapy's efficacy and reduces cardiological complications and so improving survival (Tsironi *et al.*, 2005).

Iron has also been shown to accumulate where neurons degenerate in diseases such as Alzheimer's, Parkinson's, Huntington's and amyotrophic lateral ataxia by initiating oxidative stress. There is evidence that the administration of siderophores will improve currently available treatments (Youdim *et al.*, 2004).

Moreover chelation therapy has been explored in order to minimise tissue damage, caused by iron accumulation, in patients following a heart attack (Raymond, 1994).

Iron acquisition agents can also provide prophylaxis to immunocompromised and HIV patients susceptible to develop Tuberculosis or against the activation of latent *M. tuberculosis*. Populations from Sub Sahara in Africa, where HIV, Tuberculosis, iron overload and possibly related genetic disorders regularly coexist, represent ideal candidates for this prophylactic approach (Cronje *et al.*, 2005).

Moreover the increase of *M. tuberculosis* strains resistant to drugs, currently available on the market, has urged an immediate need to develop a new more effective anti-Tuberculosis therapy. Siderophore conjugates combined with antimicrobial agents demonstrate promising results as drug delivery agents. An excellent paradigm: the incorporation of a catechol moiety into the structure of β-lactam antibiotics, such as penicillin and cephalosporin, proved to be bacteriocidal against *P. aeruginosa*. The mechanism involves the recognition and acquisition of the siderophore conjugate by a certain pathogen, due to the latter's iron transport system, and the consequent intracellular release of the metabolic inhibitor (antibiotic). Many secondary metabolites (albomycins, ferrimycins, danomycins, salmycins, nalidixic acid, vancomycin…) have been tested using this system and only a few demonstrated improved antimicrobial activities. In some cases the antibiotics completely lost their potency (Ratledge & Dover, 2000). However there is evidence that the carboxylic azanoraristeromycin trihydroxamate conjugate 22 inhibit tumour cells and display significant bacteriocidal activity against certain virus, such as reo, parainfluenza, vaccinia and cytomegalo (Ghosh *et al.*, 1999). Therefore it is worth exploring this opportunity further in order to find a metabolically stable, potentially successful, drug

with sufficient intracellular access, which not only will be effective against infections but also useful to combat antibiotic resistance.

An additional biomedical application of siderophores is the acquisition of aluminium (III) from the human body. The production of electricity generated by nuclear energy resulted in increasing exposure of the human body to transuranic elements, such as aluminium. Administration of desferrioxamines to patients has proven to be successful in terms of lowering aluminium levels in the body (Mohandass, 2005). In an earlier study conducted on mice scientists had proven that the activity of desferrioxamine as an aluminium chelator was age dependent, suggesting that the system is more efficient in younger animals than adult ones (Esparza *et al.*, 2000). Desferrioxamines have been investigated as potential entities to estimate aluminium overload. Two different doses, low (30 mg / kg) and high (50 mg / kg), have been explored and it has been revealed that low doses not only increase the sensitivity of the test itself but also demonstrate no toxic side effects (Janssen & van Boven, 1996). Additionally there is an indication that a 'cocktail' of siderophores might provide a more efficient pharmacotherapy; in terms of aluminium overload by allowing small chelating agents to penetrate the nucleus, acquire the aluminium elements and transfer it to desferrioxamines, which are slightly larger biomolecules. The proposed mechanism is well known as 'molecular shuttle chelation' (Kruck *et al.*, 2004).

Siderophores have also applications in diagnostics, where it was discovered that utilisation of ferrioxamines as the sole source of iron distinguishes the pathogen *Salmonella enterica* from a number of related species. Moreover it promotes its recovery from contaminated food (Kingsley *et al.*, 1995, Kingsley *et al.*, 1999, Reissbrodt *et al.*, 1996, Reissbrodt *et al.*, 2000).

1. 10. 2. Agricultural applications / Bioremediation:

In agriculture farmers regularly adopt chemical methods, such us pesticides, herbicides or fungicides, in order to improve crop yields. The use of biological control agents is a new, extremelly efficient approach that has not been fully explored (Nelson, 2004). The application of microbial products is not commonly used. Inoculation of crop plants with *Pseudmonas fluorescens-putida*, promotes growth and significantly icreases the yield up to 144%. The species, called plant promoting rhizobacteria (PGR), quickly colonize the roots of potato, radish and sugar beet and produce pseudobactin (siderophore), which rapidly scavanges the iron from the

environment. Therefore they compete with antagonistic, potentially deleterious fungi and bacteria by depleting iron from the environment (Kloepper *et al.*, 1980).

Heavy metals and radionuclides are known to contaminate the soil and groundwater posing a threat to both human and environmental health. Biomining microorganisms, such as *Thiobacillus*, *Alicyclobacillus* and *Acidithiobacillus* species, can absorb, reduce, oxidize, solubilize or precipitate many toxic heavy metals and radionuclides, thus impacting the speciation, solubility and ultimately the mobility of these metals (bioleaching) [Hoolden *et al.*, 2001 and Rawalings, 2002].

Siderophores also play an important role in metal recovery and remediation due to their ability to chelate metals other than Fe(III) or Fe(II), such as Mn(II), Zn(II), Cu(II), Co(II), Ni(II), Mg(II), Al (III), Ga(III), Cr(III), In(III), Sc(III) [Arceneux *et al.*, 1984, John *et* al., 2001, Kalinowski *et al.*, 2000 and Rodriguez & Smith, 2003]... In 1993 the U.S Department of Energy (DOE) identified numerous sites heavily contaminated with plutonium and uranium (actinides). More recent statements urge that the concentration of the contaminants is much higher than previously reported, posing a serious health risk. In an attempt to control the environmental catastrophe the interaction of siderophores with Pu (IV) and U(VI) was examined. *Microbacterium flavescens* (JG-9), a non-siderophore producer, was identified as an ideal candidate due to its growth dependency on hydroxamate compounds. The organism successfully acquired both Fe (III) and Pu (IV) from desferrioxamine B, demonstrating higher uptake rates for the former than the latter. In comparison accumulation of Pu(IV) appeared to be 75% less than that of Fe(III). Addition of both metals simultaneously caused the initial uptake rates of both plutonium and iron to drop by 50%, demonstrating the competition between the two metals for common binding sites. The organism failed to chelate any U (VI) from the siderophore complex, making plutonium the first tetravalent actinide to be taken up by a microorganism in combination with a chelate agent. Exploring further this application can potentially determine the fate of actinides not only in the environment but in industrially contaminated materials as well (John *et al.*, 2001).

Moreover there is evidence that siderophores have a potential application in dissolution of hornblende, a mineral abundant in granitic soils. A *Streptomyces* catechol producer has tested positive for its ability to increase the hornblende's iron release five fold compared to the standard procedure. Addition of desferrioxamine B (24μm) demonstrated a similar iron release to that observed by the presence of the *Streptomyces* isolate. The presence of both the siderophore (20-30μM) and the isolate

further increased the iron release by an extra two to three fold. This phenomenon could be explained in several different ways: desferrioxamine B a) served as a xenosiderophore, b) triggered the biosynthesis of a different siderophore due to the strain's inability to transport and acquire iron from the hydroxamate or c) converted to ferrioxamine forcing the organism to synthesise more catechol in the misconception of iron deprivation. Moreover silicon (Si) and aluminium (Al) mobilisation improved in the presence of the desferrioxamine B but not the *Streptomyces* species, indicating that catechols might display higher affinity to these elements than hydroxamates. An ideal microbial system for this approach would involve an immobilised multiple siderophore producer, able to scavenge a variety of undesirable metals from the environment (Kalinowski *et al.*, 2000 and Liermann *et al.*, 2000).

1. 11. 0. Immobilisation

Some of the most successful methods adopted for immobilisation of microorganisms have involved the use of inert supports. Furthermore particles with increased porosity, compared with those that are lesser or non-porous, have proved to be more adept at immobilising microorganisms (Yang *et al.*, 2004).

1. 11. 1. Pumice stones

Pumice is produced by volcanic eruptions and it is characterized as porous lava, which has been generated by gas bubbles that have travelled through it (Figure 16). Typically pumice is light in colour, extremely porous and may contain crystals. The stone can be elongated or rounded in shape; the latter is a consequence of abrasion that has occurred in the vent or eruption column of the volcano. It can float on water and has been known to float around the world; however it can become sufficiently waterlogged and consequently sink. Usually this process is quicker with hot pumice than cold (Cas & Wright, 1993). Pumice was successfully trialed as a support for titatanium oxide (TiO_2), which is used as a photo catalyst to eliminate organic matter from waste water (Rachel *et al.*, 2002).

Figure 16: A light colour pumice stone.

1. 11. 2. Sponge

Natural sponge has demonstrated to be a successful porous support surface for the immobilization of microorganisms, such as yeast (Ogbonna *et al*., 2001) and bacteria (Vignoli *et al*., 2006; Yang *et al*., 2004).

Inert porous and fibrous supports were trialed by using methanogenic bacteria, such as *Methanobacterium formicicum* and *Methanosarcina sp*., to increase methane production for the reduction of organic matter. The bacterial species were efficiently immobilized in the porous surfaces, namely loofah, which was deemed superior to the other supports used; methane production was also significantly increased. Furthermore it was suggested that the increase in methane production could have been a consequence of the loofah being converted to methane, as it is a natural product (Yang *et al*., 2004).

Vignoli *et al*. (2006) successfully immobilized the bacterium *Zymomonas mobilis* in loofa sponge (*Luffa cylindrica*) in an attempt to increase the production of sorbitol. *Z. mobilis* utilizes numerous fructose sources, converting them into sorbitol. It was also demonstrated that production increased twofold (Vignoli *et al*., 2006). *L. cylindrica* is a suitable candidate as it is a natural product and would not be impinging upon environmental issues such as degradation. It also does not require chemical products and immobilization is simple (Vignoli *et al*., 2006).

1. 12. 0. Final remarks:

In order to fully understand the potential value of siderophores to both humankind (chelation therapy) and the environment (decontamination), the search for new candidates must continue. Moreover certain issues have to be addressed with the intention of achieving the goal of an over- multiple biosynthesiser. Continuous systems, where such a producer cultivates under steady state conditions (pH, temperature, O_2, stirring, nutrient concentration…), would reveal vital information in terms of growth and biosynthesis; and represent the future for siderophore investigation (Hoskisson & Hobbs, 2005). Optimisation of the production media is a necessity and fed-batch fermentation should definitely be considered. Last but not least many questions have to be answered: Are the siderophores of interest biomass related? Are they intracellularly deposited or extracellularly excreted? What metals do they favour binding to and what are the mechanisms? Do organisms use particular precursors for the biosynthesis of their chelating agents? How do isolates behave under different environmental stress factors and most of all are siderophores metabolically stable or toxic to animals and human beings?

1. 13. 0. Aims of this study:

The current study is designed to achieve the following aims:

- To isolate and characterise novel siderophores produced by actinomycetes at the chemical and physiological levels.
- To identify a multiple siderophore producer and understand the mechanism of each of the specific biosynthetic pathways involved.
- Maximize siderophore production by directing its biosynthetic pathway towards the desired product using fed-batch fermentation.
- To analyse the ion acquisition and tolerance mechanisms by actinomycetes.
- To immobilise the siderophore producer on an appropriate porous surface and design a suitable system with view to improving biodegradation.

Chapter 2

Materials and Methods

2. 1. 0. Isolation of actinomycetes from soil:

The isolation of actinomycetes from soil was conducted using the soil dilution plate technique using starch-casein agar (0.4 g of casein, 1.0 g of starch, 0.5 g of KNO_3, 0.2 g of K_2HPO_4, 0.1 g of $MgPO_4$, 0.1 g of $CaCO_3$ and 15 g of agar per litre of distilled water [dH_2O] supplemented with 150 µM 2,2'-dipyridyl [Dip]). The latter is an iron chelate agent and was used in order to apply selective pressure for siderophore producers. One gram of soil sample was suspended in 100ml of sterile distilled water and incubated overnight at 40^0 C. Serial aqueous dilutions (50 µl of $10^0 - 10^{-9}$) of the soil suspension were applied onto the agar plates and incubated at 27^0 C for up to four weeks. Isolates were chosen on the basis of their hairy appearance, self limiting colonies, dark spore chains and limited life cycle, were further subcultured and pure actinomycetes were isolated (Kieser *el al.*, 2000). Finally the strains were stored in a bacterial preservation system (Technical Service Consultants Ltd, Lancashire) at - 80°C.

2. 2. 0. Siderophore production:

Siderophore production was confirmed using the Chromo Azuerol Sulphate (CAS) assay. This approach is based on the competition for iron ($FeCl_3$) between the blue CAS reagent and the chelating agent (siderophore) released by an organism. Transfer of ferric ions from the blue CAS agar to the siderophore leads to decolourisation and production of a halo, the size of which is dependent on the siderophore released. Orange halos are indicative of hydroxamate production and yellow ones demonstrate the presence of a catechol moiety (Renshaw *et al.*, 2003).

Preparation of:

2. 2. 1. CAS solution: An aliquot (50 ml) of dH_2O containing 60.5 mg of Chromo Azuerol S was mixed with 10 ml of 1mM $FeCl_3$, 10 mM of HCl in dH_2O; 72.9 mg of CTAB was added and the volume was adjusted to 100 ml. The solution was sterilised by autoclaving at 121^0 C for 15 minutes.

2. 2. 2. CAS agar: NaOH (1.2 g), PIPES (6.05 g), 20X MM9 (5 ml) [0.3 g of KH_2PO_4, 0.5 g of NaCl and 1 g of NH_4Cl were dissolved in 50 ml of dH_2O] and bacteriological agar (3 g) were mixed in 200 ml of dH_2O and autoclaved.

After autoclaving 90 ml of CAS agar was mixed with 10 ml of CAS solution and poured into plates. Agar plugs of 1 cm were removed from the CAS plates and replaced with either agar plugs containing pure cultures of actinomycetes or 50 µl of

culture supernatants (individual details of which are shown in the results section). Finally the plates were incubated at 30^0 C for 48 hours.

2. 2. 3. Modified CAS assays:

In order to investigate the siderophore's capacity to chelate various ions we developed a new modified CAS version, where the $FeCl_3$ in the CAS solution was replaced by different ions of the same molarity. These included:

- $ZnSO_4$, BDH, GPR.
- $CrCl_3$, Sigma.
- $MgSO_4$, Sigma.
- $MnCl_2$ $4H_2O$, BDH, Analar.
- $AlNH_4SO_4$, BDH, GPR™.
- $CuSO_45H_2O$, BDH, GPR™.
- $Ni(NO_3)_2$, BDH, Analar.
- $(CH_3COO)_2CO4.H_2O$, BDH.
- LiCl, Sigma.
- $CdNO_3$, Aldrich.

2. 2. 4. Arnow's Assay to detect catechol production:

The presence of a catecholic compound was further verified by Arnow's assay, which was performed by adding the following in order:

- 1 ml of culture supernatant.
- 1 ml of 0.5 M HCl.
- 1 ml of nitrite-molybdate solution: 10 g of sodium nitrite ($NaNO_2$) were added to 10 g of sodium molybdate ($Na_2MoO_4.2H_2O$) and dissolved in 100 ml of dH_2O.
- 1 ml of 1M NaOH.

The assay was allowed to incubate at room temperature for 5 minutes and the optical density was measured at 500 nm (OD_{500}) using an Uvikon 930 spectrophotometer. As a control for the Arnow's assay the supernatant was replaced by 1 ml of dH_2O.

Catecholic compounds produce a yellow colour when are combined with nitrous acid, which becomes a pink-red colour (depending on the intensity) in the presence of excess NaOH (Arnow, 1937).

2. 2. 5. Atkin's Assay to detect hydroxamate production;

Hydroxamate producers were determined applying the colorimetric assay of Atkin's: 500 µl of culture supernatant were added to 2.5 ml of Atkin's solution (0.1771 g of $Fe(ClO_4)_3$ was dissolved in 100 ml of dH_2O after the addition of 1.43 ml of $HClO_4$. The assay was allowed to incubate at room temperature for 5 minutes and the optical density was measured at 480 nm (OD_{480}) using an Uvikon 930 spectrophotometer. As a control for the Atkin's assay the supernatant was replaced by 500µl of water. Orange colour indicated the presence of hydroxamate (Atkin, 1970).

2. 3. 0. Detection of hydroxamates by Thin Layer Chromatography (TLC):

Liquid cultures of microorganisms were sampled (1 ml) and centrifuged at 13000 rpm for 10 minutes at 4^0 C. The supernatant was removed, 5 µl of 1 M $FeCl_3$ was added and the solution was mixed by inversion. Prior to use the silicon plate (Polygram^R SIL, G/UV_{254}) was preheated for 5 minutes at 50^0 C. Aliquots (1-5 µl) of the ferric supernatants were spotted onto the TLC plate alongside desferrioxamine B, E, and ferrichrome B standards and placed in a TLC tank containing Methanol/dH_2O (2:1, v/v) as the mobile phase. Plates were left to run for approximately 15 minutes and the coloured hydroxamates were visualised as orange/brown spots.

2. 4. 0. Detection of ferrioxamines by High-Performance Liquid Chromatography (HPLC):

Ferrioxamines were examined using a HPLC (Biorad Model 2700) equipped with a SphereClone 5µ ODS (2) [150 x 4.60 mm column]. The mobile phase consisted of 0.1 % v/v phosphoric acid (A) and 100 % v/v acetonitrile (B) delivering the solvent A to the HPLC system at a concentration of 95 % - 75 % of solvent A at a flow rate of 1.5 ml/min for 20 minutes. The sample volume was 20 µl of the filtered ferric culture supernatant (5 µl of $FeCl_3$ per ml of supernatant) and the effluent was monitored at 435 nm by a Biorad-Bio-dimension^{TM} UV/VIS equipment.

2. 5. 0. Catechol extracts and TLC analysis:

Catechol containing supernatants, obtained by centrifugation of 1ml of selected cultures at 13000 rpm (Eppendorf microcentrifuge) for 10 minutes at 4^0 C, were acidified to pH 2.0 by addition of 6 M HCl. Acidification of the medium allows for protonation of any ferric siderophore complexes and the subsequent release of the iron molecules. The catechol was extracted by addition of 1 ml of ethyl acetate, forcing the compound to migrate from the aqueous phase to the solvent, which was recovered and air-dried. The concentrated product was resuspended in a minimum amount of ethyl acetate (5 µl maximum). TLC of the ethyl acetate extracts was performed on preheated (5 minutes at 50^0 C) silicon plates (Polygram^R SIL, G/UV_{254}) and left to develop in a TLC tank for 15 minutes in the presence of methanol/ chloroform (1:1, v/v) as the mobile phase. Compounds were visualised by fluorescence under a UV source.

2. 6. 0. Strain characterisation:

In order to establish the identity of the strains, screened for siderophore production, a variety of characteristics were determined to produce a detailed phenotypic and metabolic profile. This approach provided key information about the phylogenetic relationships and growth requirements of the isolates.

Actinomycete colonies were characterised following directions given by Bergey's Manual of Systematic Bacteriology (Locci, 1989); cultural and morphological characteristics were observed by light and scanning electron microscopy.

The ability of the organisms to utilise different carbon sources was tested using Biolog^{TM} plates SF-N2 (1807291) and GP2 (1811021), purchased from OXOID Ltd., Basingstoke, Hampshire, England. These plates provide an easy and rapid metabolic testing of sporulating and filamentous microorganisms, such as actinomycetes. The procedure involved the addition of 100 µl of spore stock (OD_{590} of 0.022) in each well (96 in total) and the incubation at 30^0 C for 3 to 7 days. Positive reactions were determined either by a purple colour due to the presence of a tetrazolium redox dye (GP2) or increased turbidity (SF-N2).

The strains were also tested for their ability to grow and sporulate on a range of liquid or solid media, including yeast extract-malt extract (YEME), Mannitol-Soya (MS), Muller-Hinton (MH), MH^+ glucose, MH^+ fructose and Starch-Casein agar. The effect of pH and temperature on their growth patterns was also investigated.

2. 6. 1. Muller-Hinton (MH) agar*:

MH agar (38 g) [OXOID] was suspended in 1 litre of dH_2O and the solution was sterilised by autoclaving at 121^0 C for 15 minutes. MH glucose and MH fructose agar included per litre 5g of glucose and 5g of fructose respectively.

2. 6. 2. MS solid and liquid cultures*:

D-mannitol (20 g), soya bean flower (20 g) and bacteriological agar (20 g) [OXOID] were dissolved in 1 litre of dH_2O and sterilized by autoclaving. MS liquid media were exactly the same with the bacteriological agar omitted. An aliquot of 50 ml of the medium was distributed in 250 ml shake flasks prior to sterilization.

2. 6. 3. YEME solid and liquid cultures*:

Yeast extract (4 g), malt extract (10 g), $_D$- (+)-glucose (4 g) and bacteriological agar (20 g) were dissolved in 1 litre of dH_2O and sterilized by autoclaving. Liquid YEME media were exactly the same with the bacteriological agar omitted. An aliquot of 50 ml of the medium was distributed in 250 ml shake flasks prior to sterilization.

*Wherever necessary solutions containing different concentrations of either amino acids or $FeCl_3$ were filter sterilised using Ministart^R filter units, (0.20μm, Sartorius) prior to inoculation into the solid or liquid media.

2. 6. 4. 0. Difco 547 ISP4 agar

Starch (20 g), $CaCO_3$ (4 g), K_2HPO_4 (2 g), $MgSO_4.7H_2O$ (2 g), NaCl (2 g), $(NH_4)_2SO_4$ (4 g), agar (40 g) and 2 ml of trace salts solution were dissolved in 1litre of dH_2O and sterilised by autoclaving following pH adjustment at 7.0 - 7.4 (Williams *et al.*, 1982).

Trace salts solution

$FeSO_4$ (0.1 g), $MnCl_2$ (o.1 g) and $ZnSO_4$ (0.1 g) were dissolved in 100 ml of dH_2O.

2. 6. 5. Antibiotic resistance:

Antibiotic resistance analysis is commonly employed as a taxonomic tool. This particular characteristic of the strains was examined using susceptibility discs (OXOID, MAST) performing the disc diffusion assay. An aliquot (50µl) of a spore stock (OD_{590} of 0.2) was spread on starch casein agar plates and the susceptibility discs were placed upon the plates prior to incubation at 4^0 C overnight. Finally the plates were incubated at 30^0 C for 4-7 days.

2. 7. 0. Culture viability:

Culture viability was monitored using fluorescence microscopy and the BacLight™ stain (Cell Viability Kit; Molecular Probes), which colours viable cells green and those with compromised membranes red. Optimum staining was achieved by mixing 1.5 µl of SYTO 9, 1.5 µl of propidium iodide and 500 µl of deionised H_2O. Samples were prepared by mixing 2.5µl of the stain with 2.5 µl of cell suspension onto which was placed a coverslip. The pellets were visualised using an Olympus BX51 microscope and the pictures were captured by an Olympus camera (QIMAGING, MicroPublisher 3.3 RTV, U-CMAD3).

2. 8. 0. Effects of ions on cell morphology

Agar plates (MS and starch casein) were inoculated to confluence and centre wells (1cm diameter) were cut out and a 100 µl of an appropriate ion concentration was added. Growth and cell morphology was investigated following three-day incubation at 30^0 C.

2. 9. 0. Extraction of genomic DNA (Kirby mix procedure):

The 'Kirby mix procedure' is a phenol/chloroform extraction and is commonly employed for the isolation of genomic DNA from actinomycetes:

Mycelia from a 25 ml cultures (48 hours old) were resuspended in 3mls of TE25S buffer (25 mM Tris-HCl, pH 8, 25 mM EDTA pH 8, 0.3 M sucrose) and 100 µl of lysozyme were added. Final lysozyme solution was 2 mg/ml. An aliquot (4 ml) of 2x Kirby mix (2 g of SDS, 12g of sodium 4-aminosalicilate, 5 ml of 2M Tris-HCl pH 8, 6 ml of equilibrated phenol pH 8 dissolved in 100 ml of dH_2O) was added and the solution was agitated for 1 min on a vortex mixer. An aliquot of 8 ml of phenol/chloroform/isoamyl alcohol (50 ml equilibrated phenol, 50 ml of chloroform, 1 ml of isoamylalcohol) was added and the mixture was agitated for 15 minutes as above, following centrifugation for 10 minutes at 3500 rpm. The upper phase (aqueous) was transferred to a fresh eppendorf containing 3ml of phenol/chloroform/isoamyl alcohol and 600 µl of 3 M unbuffered sodium acetate following agitation and centrifugation as in step 2 and 3 respectively. The solution was mixed in the presence of 0.6 volume of isopropanol, the DNA was spooled onto a sealed Pasteur pipette and washed in 5 ml of 70 % ethanol. The pellet was redissolved in 5ml of TE buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA) and 50 µl RNAse A (4 mg/ml) prior to incubation at 37^0 C for 30 minutes. Steps 4 and 5 were repeated and finally the pellet was air dried and redissolved in 0.5-1 ml of TE buffer.

The presence of genomic DNA was verified using 1 % w/v agarose gel electrophoresis.

2. 10. 0. 16S rRNA amplification and detection of PCR products:

Ribosomal RNA (rRNA) is the most conserved gene in all cells and its characteristics are important to evolution and phylogenetic analysis. Amplification of the 16S rRNA sequences were performed with a Perkin-Elmer Cetus GeneAmp Thermal Cycler, 9600, using 35 cycles of 95^0 C for 1 minute, 52^0 C for 1 minute and 72^0 C for 2 minutes. Reaction mixtures contained 45 µl of 1.1 x PCR MasterMix (1.5 mM $MgCl_2$) [ABgene], 2 µl of DMSO, 1 µl of genomic DNA (50 ng), 1 µl of sterile dH_2O and 0.5 µl of each primer (forward and reverse). In order to achieve maximum sequencing of the 16S rRNA genes three sets of Streptomyces-specific primers were used: Strep B- StrepE, StrepB- StrepF, Fd1-Rd1 with the sizes of the PCR amplification products expected to be of 519 bp, 1074 bp and 2026 bp respectively

(Rintala *et al.*, 2001 and Weisburg *et al.*, 1991) [Table 7]. The amplified products were visualised on an ethidium-bromide-stained 2 % w/v agarose gel prior to purification with the QIAquick^{R} PCR purification kit (50) [Qiagen].

Primer	Sequence (5' to 3')
StrepB (forward)	ACAAGCCCTGGAAACGGGGT
StrepE (reverse)	CACCAGGAATTCCGATCT
Strep F (reverse)	ACGTGTGCAGCCCAAGACA
Fd1 (forward)	AGAGTTTGATCCTGGCTCAG
Rd1 (reverse)	AAGGAGGTGATCCAGCC

Table 7: Streptomyces specific primers used for the amplification of 16S rRNA gene (Rintala *et al.*, 2001 and Weisburg *et al.*, 1991).

2. 11. 0. Taxonomic grouping:

PCR products were sent to Lark$_{TM}$ Technologies, Inc (United Kingdom) according to their requirements. The sequences obtained were individually checked for errors and manually aligned on a Word document. Pairwise sequence comparisons and retrieval of homologous sequences were conducted using the NCBI BLAST database (available online- http://www.ncbi.nih.gov/). Similar sequences, including an outgroup (ancestral sequence), were multiple aligned by BioEdit sequence alignment Editor 7.0.4.1. (Copyright 1997-2005, Hall, 1999). Taxonomic analysis was performed by the ClustalX software, version 1.83 and Bootstrap confidence values (1000) were provided by the Neighbour- Joining (NJ) algorithm. The latter converts the DNA sequences to a model representing the evolutionary distances between the related sequences and that of outgroup's, which roots the tree and provides information about ancestral sequences. The matrix involves sampling of 1000 alignment sites (depending on the user's instructions), construction of 1000 trees and the final calculation of the appearance value of each group. It is a common and rapid approach suitable for processing related sequences. The algorithm is relatively poor for estimating evolutionary distant relationships due to the lack of accuracy, which could lead to loss of minor changes (deletions, insertions...) that might have occurred along the branches (Holder & Lewis, 2003). Finally the phylogenetic tree was presented using TreeView (Win 32) 1.6.6 (Copyright 2001 by Roderic. D. M. Page, available online: http://taxonomy.zoology.gla.ac.uk/rod/rod.html).

2. 12. 0. Standard operating procedure for fermentation process:

The most efficient method for production of siderophores by fermentation was found to be by fed batch culture (Meiwes *et al.*, 1990). The standard protocol employed in the laboratory was as follows:

Spores of the Streptomycetes species stored in bacterial preservation system (Technical Service Consultants Ltd, Lancashire) at - 80° C (10 µl) were spread onto starch casein agar plates and incubated at 30^0 C for a week until sporulation occurred (cultures became grey). Fresh spores were collected aseptically (in 20 ml of sterile dH_2O) in the flow cabinet (class 2) and an aliquot (100 µl) were introduced into 250 ml conical flasks containing 50 ml of "Preculture Medium" (MS) prior to incubation in a rotary shaker (200 rpm) at 30^0 C for 48 hours. These cultures were used for inoculating aseptically the bioreactor containing 5 litres of autoclaved "Production Medium" (MS, YEME or starch casein liquid media depending on the circumstances). Prior to inoculation of the bioreactor lysine (5 g/L) was dissolved in 200 ml of distilled water (dH_2O), autoclaved (15 psi for 15 min) and then added to the bioreactor through a peristaltic pump. The bioreactor conditions were: Temperature at 30 or 37^0 C, pH at 7.5 controlled by addition of 1.5 M NaOH and 1.5 M H_2SO_4, aeration of 1.0-v/v/m and a stirring rate of 500rpm.

2. 12. 1. Feed:

Lysine (12 g/L) and sucrose (5 g/L) were each dissolved separately in 200 ml of dH_2O, autoclaved separately (15 psi for 15 min) and then fed to the bioreactor through a peristaltic pump. The feed regime adopted (apart from some exceptions mentioned in the results section) was to add 50 % of the feed 24 h after bioreactor inoculation and then the remaining 50 % 6 days post inoculation. The fermentation was harvested after 10 days.

2. 13. 0. Immobilisation

2. 13. 1. Pumice stones

Pumice stones (grey) were collected from the volcanic island of Santorini, Aegean Sea, Greece. They were collectively broken and sized between 4 mm-5.6 mm using 2 lab sieves (size 4 mm and 5.6 mm). They were further washed with dH_2O and dried. Various coloured pigments, brick red, brown stone (found within the pumice), yellow, green, orange-red, and grey with black spots, were observed in the grey pumice stones. Samples of each coloured stones were selected and evaluated for the suitability to immobilise the actinomycete.

2. 13. 2. Immobilizing surfaces in liquid cultures

Immobilizing agents (2.5 g) were placed into 250 ml flasks, containing 50 ml of the appropriate media (pH 7). The flasks were autoclaved and inoculated with 200 µl of an aliquot of 23F. The cultures were incubated at 250 rpm and 30° C. The stones were observed under the dissecting microscope (Kyowa Optical Model, SDZ-PL) and the scanning electron microscope (SEM, Jeol JSM840) in order to determine the presence of the organism on their surface. Two stones from each sample were also subjected to Gram stain. A control was also introduced for comparison studies.

2. 13. 3. Scanning electron microscopy (SEM)

Immobilizing surfaces, such as pumice stones, were preserved in a gluteraldehyde solution (2.5 %) overnight, rinsed in 10 equal volumes of dH_2O and kept in dH_2O for an hour following the same procedure twice. The stones were removed from the water and left to dry for SEM analysis.

2. 13. 4. X- ray analysis

Selected samples were analysed under the SEM using the 'Spirit' image-capture system (Princeton Gamma-Tech (PGT)) and software. X-ray analysis [Energy Dispersive Analysis by X-ray (EDAX)] was performed using a Si-Li detector, which was kept cold with liquid nitrogen. The count rates were in the range of 231 to 3522 for all of the samples. An accelerating potential of 20 KV and a beam current of 2 mA were also applied.

2. 13. 5. Bioreactor Trial for immobilised cells

A model bioreactor was designed that incorporated a 2 L vessel with filtered (0.2 μm) inlet and outlet ports for gas exchanges (Figure 17). Additionally a sample and inoculation port was provided and the system was also equipped with a constant air (oxygen) supply, maintaining a steady oxygen deliver. The vessel was also heated at 30^0 C and stirred as appropriate.

Inert surfaces and the appropriate media were introduced into the reactor, sterilised by autoclaving and following inoculation (species: 23F) samples (5 ml) were collected daily for the CAS assay analysis.

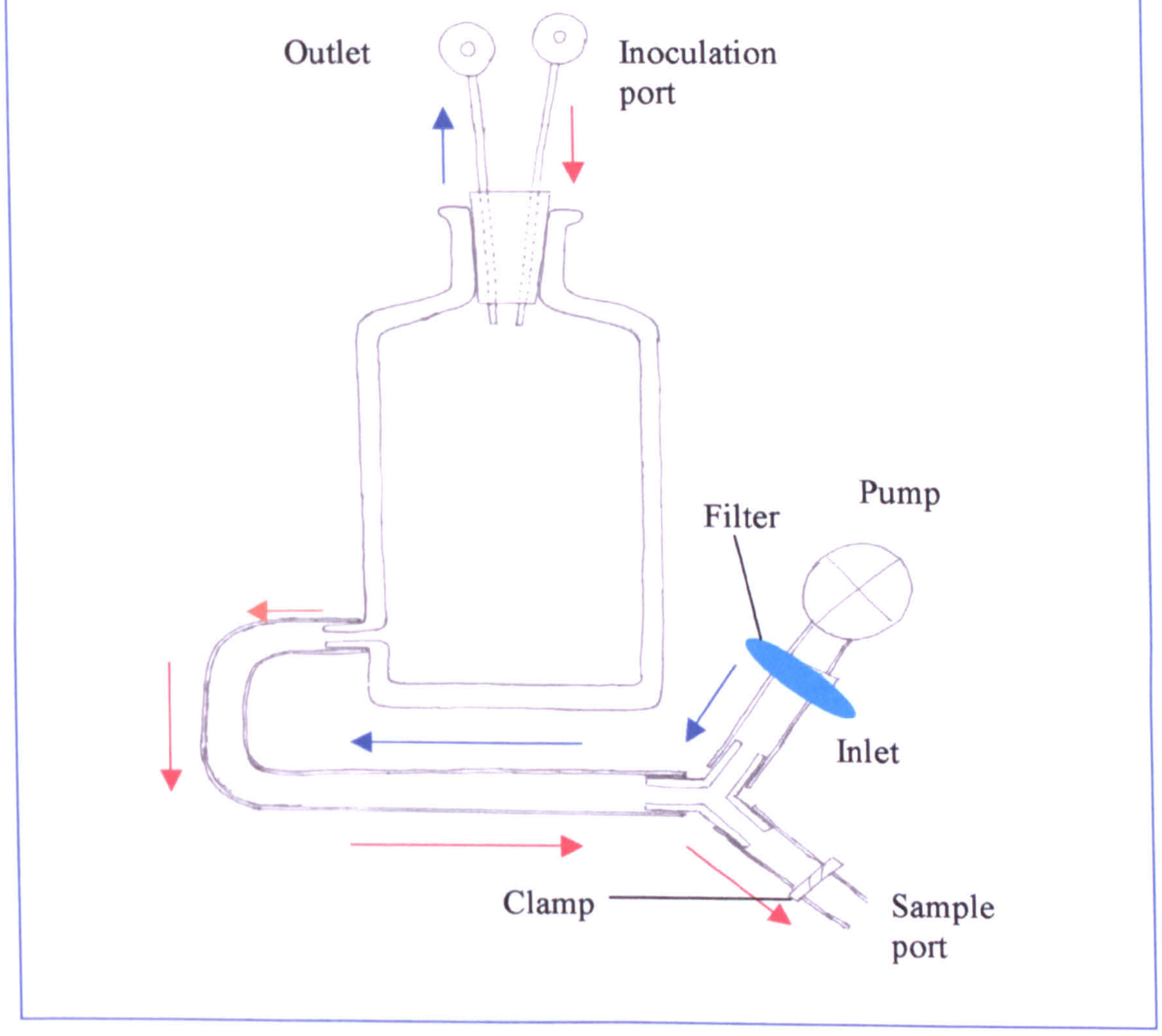


Figure 17: Schematic diagram of the bioreactor model. Airflow: →

Sample and medium flow: →

Chapter 3

Results and Discussion

3. 1. 0. Screening for siderophore producers:

In an attempt to identify novel siderophore compounds and producers we have isolated and purified 154 cultures from soil samples collected in Thailand (Tak province) and the UK (Freshfield). The majority of colonies are considered to belong to actinomycetes on the evidence of the specific media applied for their isolation with a further verification by phenotypic examination under the dissecting microscope (self-limited colonies, hairy appearance, substrate-aerial mycelia, pigmented spores). The actinomycetes are notorious environmental scavengers and have well-developed processes to capture ions in conditions depleted of elements; they are therefore an ideal target group for exploitation in ion binding technology (Bentley *et al*., 2002, Challis & Ravel, 2000 & Meiwes *et al*., 1990).

All the isolates were grown on starch casein agar containing 150 μM 2,2'-dipyridyl (Dip), a powerful iron-chelating agent, which scavenges any ferric complexes from the environment and we have used it to apply selective pressure for organisms adapted to iron deficiency. Twenty-two Thai and five UK strains were confirmed CAS-assay positive, in some cases exhibiting prolific siderophore production observed as large halos (Figure 18). Moreover the strains' ability to survive in iron-limited conditions was determined by inoculating them into media containing up to 600 μM Dip. Those strains that showed growth after 10 days (maximum) were considered to be well adapted for growth in an iron deprived environment. Nevertheless there were cases that some organisms demonstrated late (20-30 days) and very poor growth especially in the presence of 400-600 μM Dip. Certain actinomycetes from the laboratory strain collection were also included in this experiment for comparative studies (Table 8, page 64-65).

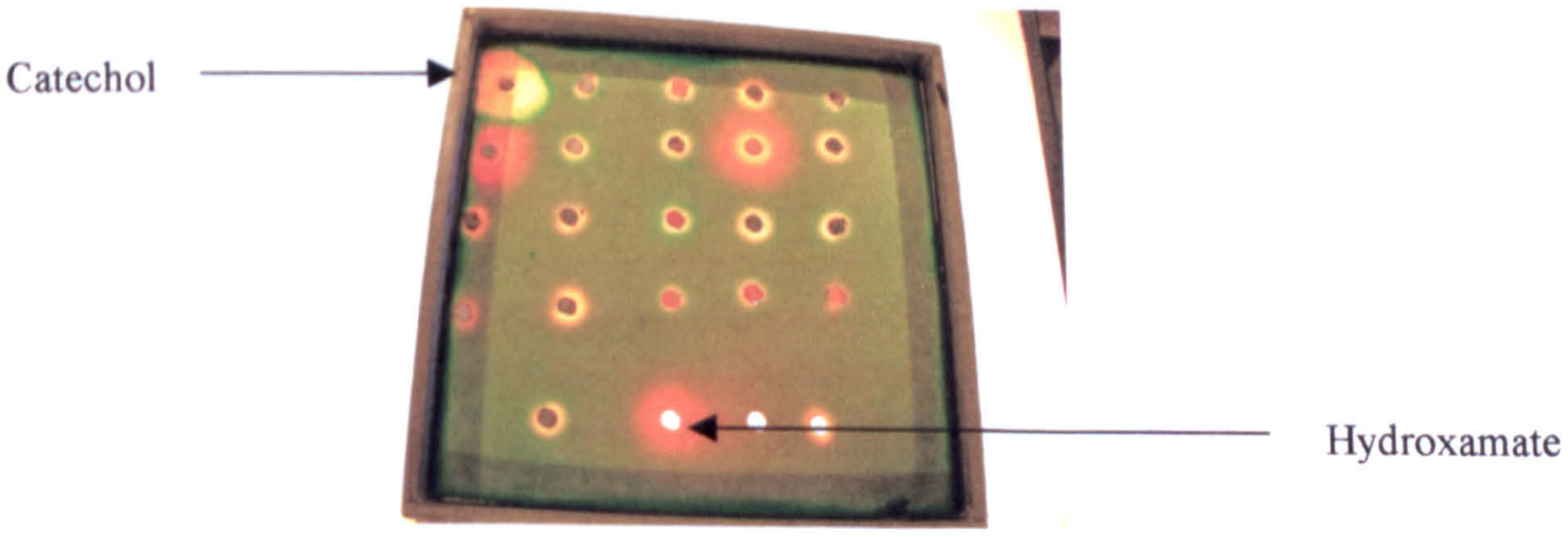


Figure 18: A typical example of a CAS assay. It is worth noticing that both catechol (yellow halo) and hydroxamate (orange halo) producers were identified.

3. 1. 1. Siderophore characterisation:

In an attempt to categorise the siderophores produced by the microorganisms, two rapid colorimetric assays were employed. Atkin's assay for hydroxamates and Arnow's assay for catechol siderophores were applied to culture supernatants from various liquid grown cultures. A typical result for catechol identification is shown in Figure 19.

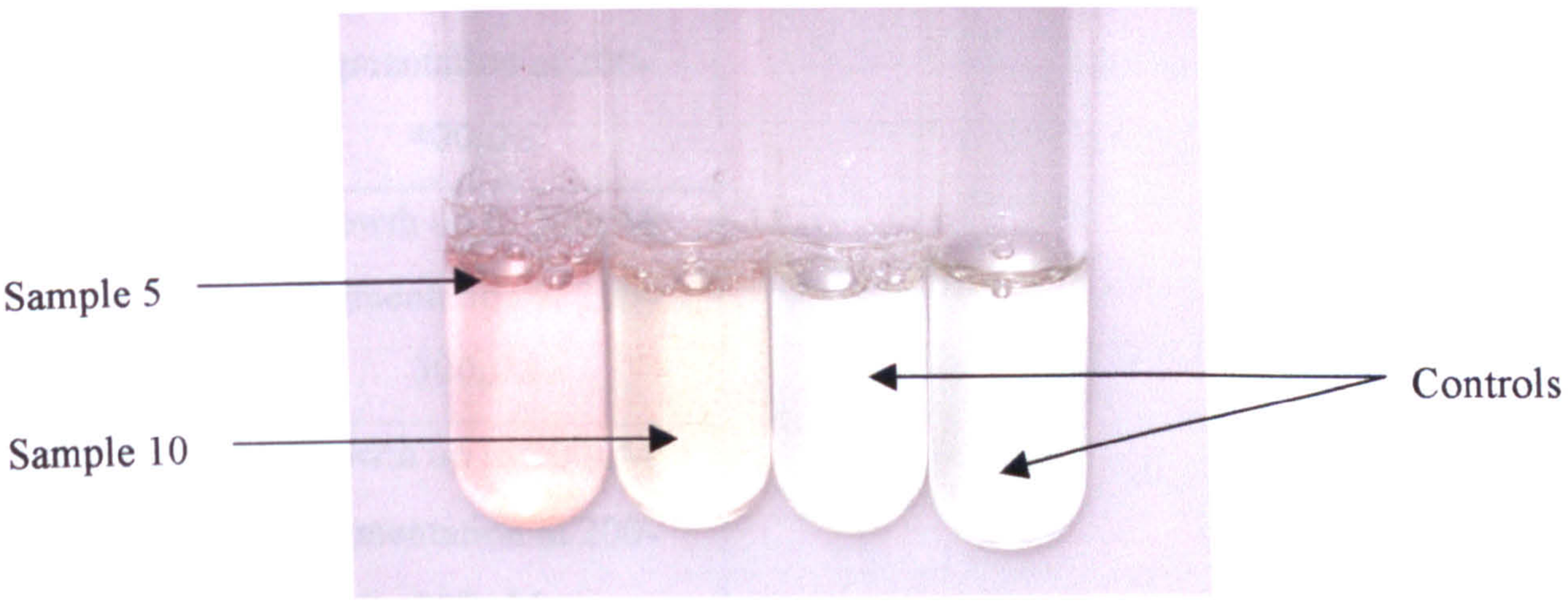


Figure 19: A successful Arnow's assay (for catechol identification). Samples 5 and 10 from Freshfield were screened positive for catechol production (pink colorization). The last two samples are non-producers (controls).

Both of the assays were successfully performed on stains 5 and 10 from Freshfield, interestingly revealing our first two multiple producers (Table 8). These results confirmed the contingency theory, which proposes that members of streptomycetes produce multiple siderophores in order to overcome the biological competition in nature (Challis & Ravel, 2000). Care had to be taken when interpreting the Atkin's assay as in some cases the culture medium was highly pigmented (Figure 20).

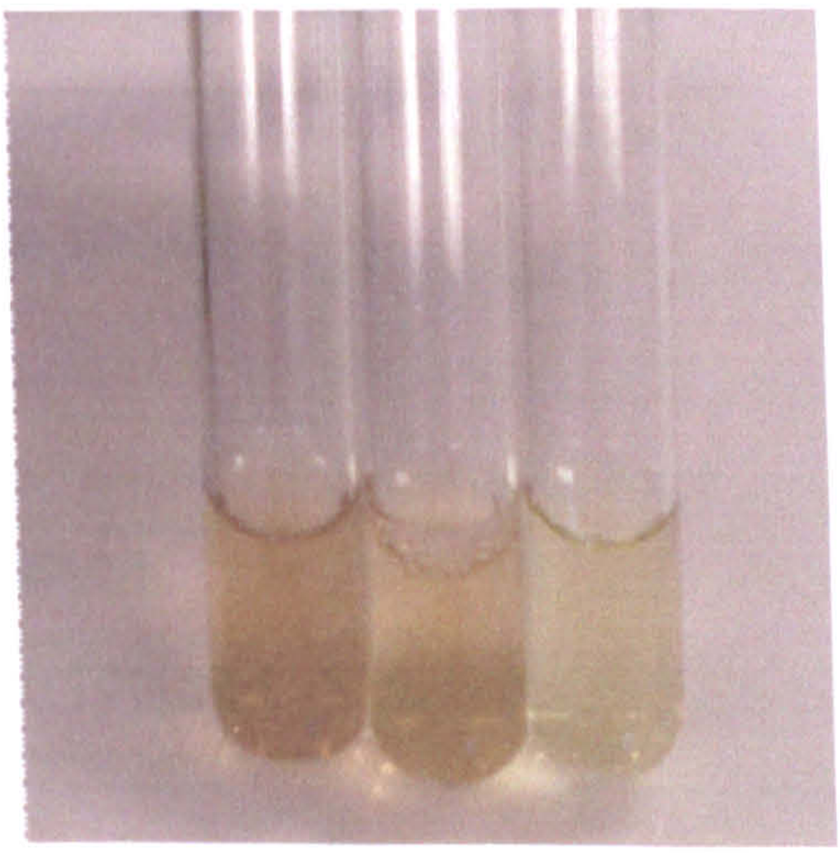


Figure 20: An inconclusive Atkin's assay of Thai isolates 31B, 31C and 33D due to the presence of yellow/brown pigment in the media.

Organism	Dip concentration	Isolated from:	CAS assay	Siderophore produced:
5*	LG between 500-600μM. Pigmentation at 200-400μM	Freshfield soil	+	Hydroxamate/ Catechol
6	LG between 500-600μM. Pigmentation at 200-400μM		+	?
10*	Growth up to 300μM Pigmentation at 200-300μM		+	Hydroxamate/ Catechol
15	Growth up to 300μM. Pigmentation at 200-300μM		+	Catechol
34	LG between 450-500μM. Pigmentation at 200μM	Freshfield sandy soil	+	?
1A	Growth up to 300μM	Soil sample, dipterokarp forest, Tak province, Thailand	+	?
1B	LG at 400μM		+	Overproducer
5A	-		+	?
8B	Growth up to 400μM		+	Hydroxamate
8C	Growth up to 300μM		+	?
1L	-		+	Overproducer
2B	-		+	?
2C	-		+	?
2D	Growth up to 100μM		+	?
1L	-		+	?
5A	-		+	?
1J	LG at 400μM		+	?
18E1	Growth up to 300μM		+	?
18E2	-		+	?

23F	Growth up to 600μM	Soil sample from Taksinmaharat National Park, Thailand	+	Overproducer
26B	-		+	?
28D	-		+	?
28B	-		+	?
29C	Growth up to 150μM		+	Catechol overproducer
31B	Growth up to 150μM	Soil sample, dipterokarp forest, Tak province, Thailand	+	Catechol Overproducer
31C	Growth up to 150μM		+	Catechol Overproducer
33D	Growth up to 150μM		+	Overproducer
Amycalotopsis abba	LG at 600μM	Laboratory strain collection	ND	?
A. azurea	LG at 600μM. Pigmentation at 450-500μM.		+	?
A. fastidiosa	LG at 600μM.		ND	?
A. japonica	Growth up to 450μM.		ND	?
A. methanolicus	LG up to 600μM		ND	?
A. orientales	LG up to 600μM		ND	?
A. thermoflosa	Growth up to 450μM		ND	?
*S. coelicolor**	LG at 600μM.		+	Hydroxamates
S. griseus	LG at 600μM.		+	?
*S. olivaceous**	Growth up to 550μM		+	Hydroxamates

Table 8: A summary of isolates obtained from Freshfield and Thai soil samples, their ability to produce siderophores and tolerate Dip compared with other known actinomycetes (Starch casein media). LG stands for Light (usually late) Growth and * indicates multiple siderophore synthesisers. ND stands for not determined. Where the term overproducer appears without qualification it was unclear from the data whether the strain was a multiple producer or not. Where ? appears the isolate produced a siderophore but due to culture pigmentation it was not possible to clarify the product using either Atkin's or Arnow's assays.

Characterisation of all the siderophore synthesisers had proven not only time consuming but also cost ineffective. On the basis of their prolific siderophore production strains 23F (unknown chelating agent producer), 29C (catechol producer), 33D (unknown siderophore producer), 31B (catechol producer) and 31C (catechol producer) were selected for further study.

3. 2. 0. 16S rRNA gene sequence analysis and phylogenetic studies:

One of the aims of the study was to have a robust and well characterised organism for the production of siderophores. On the way to this goal it was seen as important to be able to classify the organism with view to protecting intellectual property rights. 16S rRNA gene sequence analysis was carried out to elucidate the taxonomic position of the selected isolates. Almost complete 16S rRNA sequences of the representatives were determined following the isolation of genomic DNA and sequencing of the amplified genes (Appendix I). The levels of 16S rRNA sequence similarities between the isolates and actinomycetes species ranged from 97 - 99 %, confirming that the strains are members of the family and mainly appeared to be streptomycetes (Figure 21) [Table 9].

The high percentage similarity values amongst species of the same genus are very common due to the highly conserved nature of the 16S RNA gene. For example, although *S. macrosporus* and *S. megasporus* share 98 % similarities, they are clearly different on their phenotypic characterisation. A second paradigm includes *S. thermocarboxydovorans* and *S. thermodiastaticus* (99 %) or *S. thermocarboxydovorans* and *S. thermoviolaceous* (98 %) [Kim *et al.*, 1998]. Moreover *A. albidoflavus* share 97.4 % similarities with *A. azurea* and *A. orientalis* (Lee & Hah, 2001).

Although 16S rRNA analysis is a very powerful tool in phylogenetic relationships, it does not provide enough discrimination for classification of closely related species. Therefore a 'universal' approach, which will include the combination of modern molecular techniques with a number of morphological, physiological and biochemical characteristics, will facilitate the understanding of the genotypic and phenotypic signature of these isolates.

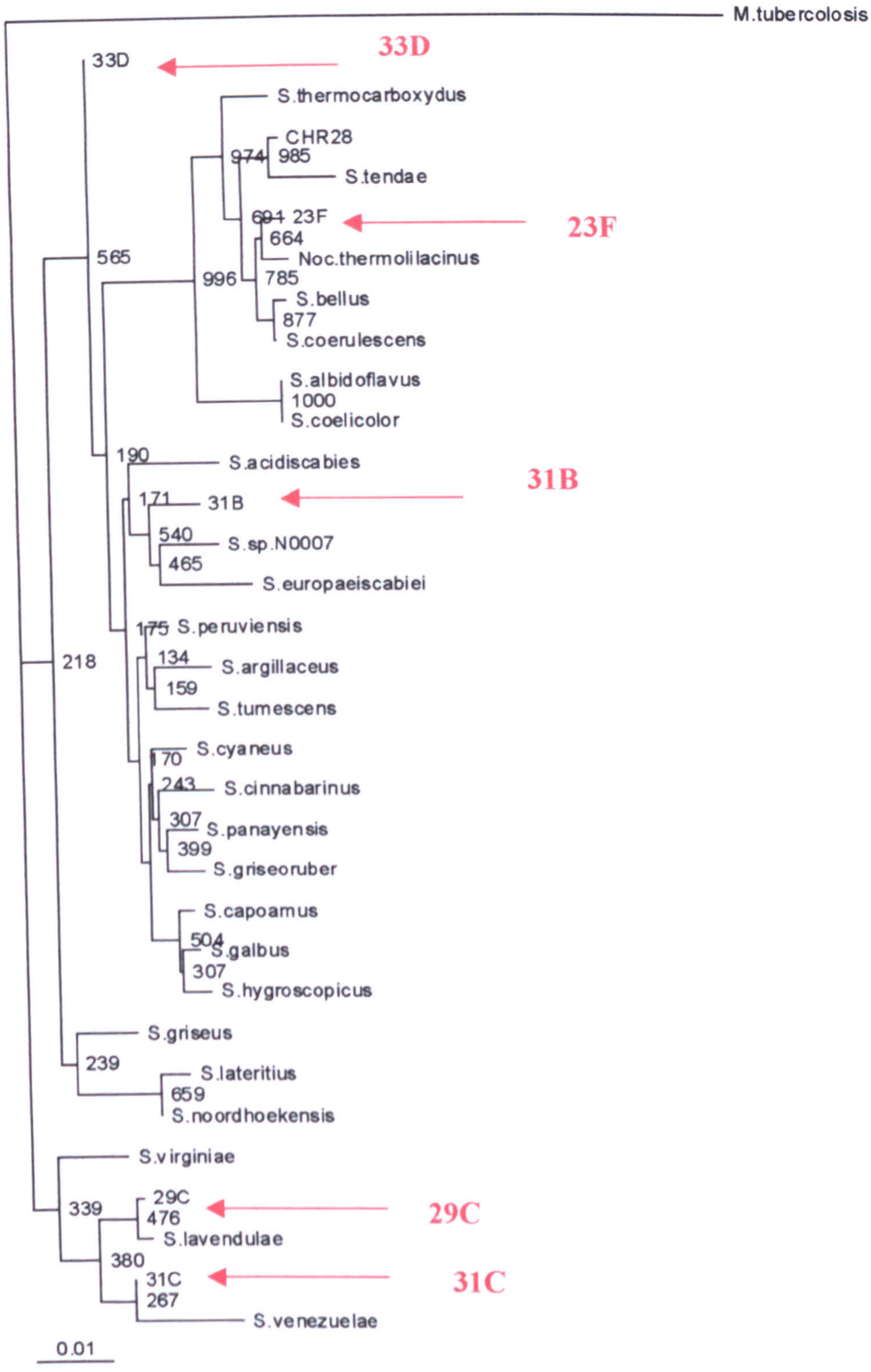


Figure 21: Phylogenetic relationships of the isolates and related Actinomycetes based on the 16S rRNA sequence analysis. The tree is bootstrapped by 1000 times by Neighbour-Joining (N-J).

Isolates									
29C		23F		31B		31C		33D	
Actinomycetes sp.	Similarity (%)	Actinomycetes sp.	Similarity (%)	Actinomycetes sp.	Similarity (%)	Actinomycetes sp.	Similarity (%)	Actinomycetes sp.	Similar (%)
S. lavemulae (IFO-14028)	99	*S. bellus* (ISP5185)	99	*S. capoamus*	98	*S. polychromogenes*	99	*S. bungoensis*	99
S. lavenulae (12340)	98	*S. thermocarboxydo-vorans*	98	*S. bungoensis*	98	*S. lavendulae*	99	*S. longwooden-sis*	99
S. lavenulae- (3125)	98	*N. thermolilacinus*	99	*S. longwoodensis*	98	*S. flavotricini*	99	*S. galbus*	99
S. lavenulae- (12344)	98	*S. coeruleorubidus*	98	*S. galbus*	98	*S. tanashiensis*	99	Clone AKIW619	99
S. lavenulae- (13709)	98	*S. speibonae*	98	Sp.N0007	98	*S. rubiginosohelvolus*	99	*S. capoamus*	99
S. griseus- (ATCC10137)	98	*S. thermotolerans* (ISP5227)	98	*S. chrestomycetic us*	98	*S. venezuelae*	98	*S. tumescens* CU21	99
S. clavuligerus	98	*S. hawaiensis*	98	*S. cyaneus*	98	*S. globosus*	98	*S. longisporus*	98
S. latericus (CSC113.1)	98	*S. purpurascens*	98	*Streptomyces corchorusii*	98	Sp. KJ-72	98	*S. tumescens* Km-1-1	99
S. virginiae- (13161)	98	*S. tendae*	98	*S. panaciterrae*	98	*S. cinnamonensis*	98	*S. tumescens* CO-7	99
S. virginiae- (15894)	98	*S. aureofaciens*	98	*S. flavovariabilis*	98	*S. spororaveus*	98	*S. flavovariabilis*	99
S. virginiae- (3729)	98	*S. goraiensis*	98	*S. peruviensis*	97	*S. nojiriensis*	98	*S. peruviensis*	98
S. virginiae- (12827)	99	*S. thermocarboxydus*	98	*S. panayensis*	97	*S. exfoliatus*	98	*S. cyaneus*	98

Table 9: The isolates' similarities with related species.

3. 3. 0. Characterisation of the strains:

All the selected streptomycete strains required further investigation in order to characterise their physiology, nutrient requirements, optimum growth conditions, antibiosis and siderophore overproduction (Table 10, page 76-82).

The Biolog microplates were employed in order to determine the 'metabolic fingerprint' of the organisms that could reveal valuable information regarding specific media design and special culture conditions.

Antibiotic resistance data provide useful information on the characteristics of the cells. A recent report by Hopwood, 2007 reaffirms the importance of actinomycetes as a major reservoir of antibiotic resistance genes. Clearly these characteristics can be a useful way of classifying strains. This could be a valuable evolutionary route to differentiate strains within a population. Given the fact that *Streptomyces* have been found to have the capacity to produce multiple "antimicrobial" compounds (Bentley, 2002 & Omura *et al.*, 2001) and hence the ability to be resistant to these compounds, it will be interesting to develop a resistance fingerprint system for strains and to revisit phylogenetic analysis in the light of this information.

3. 3. 1. Description of 29C (GenBank accession number EF585407):

Strain 29C is a Gram-positive, aerobic, white mycelia-forming organism that undergoes the typical streptomycetes growth cycle resulting in the production of pink spirales spores (Figure 22). Spores were only observed on starch-casein and YEME solid media. Examination by SEM revealed the spore surface to be smooth. It did not sporulate on MS (Mannitol-Soya bean flower) agar but it produced a diffusible brown pigment (presumably melanin).

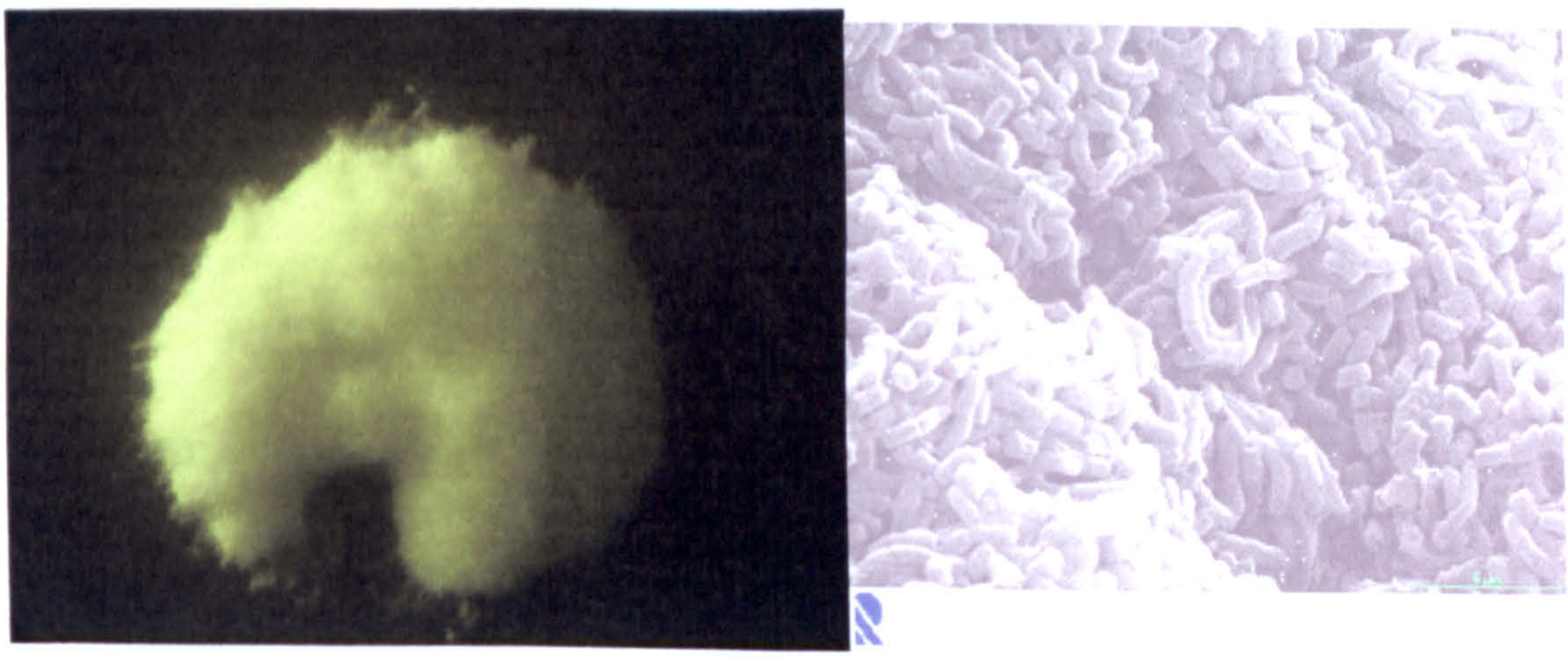


Figure 22: Light (left) and scanning electron microscopy image (right) [magnification 5000x] of 29C. In both cases cultivation was on starch-casein agar.

It tolerated only 150μM Dip (Table VIII), nevertheless overproduced a very powerful catechol based siderophore that formed strong ferric complexes in starch-casein media (Figure 23). It was found to be bactericidal against *Bacillus subtilis* and *Candida albicans* under all conditions tested. Its optimum growth required cultivation in starch-casein media, pH 7.5 at 30^0 C and it was a very slow grower.

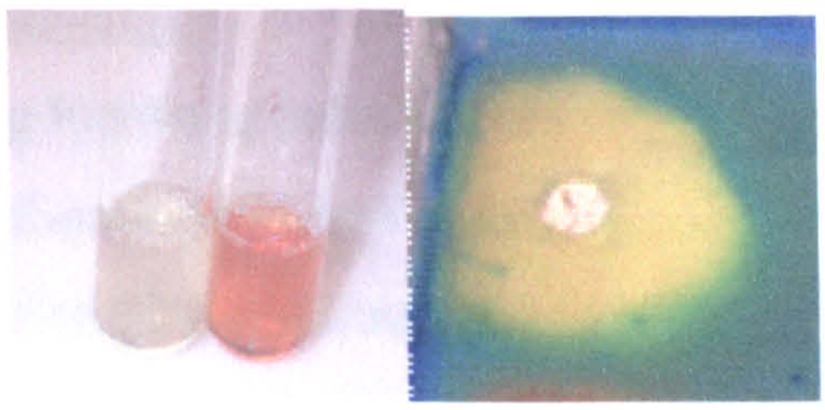


Figure 23: An Arnow's (catechol production) positive reaction (left) of 29C growing on starch-casein liquid media confirmed by CAS assay as well (growth on starch-casein agar). It is worth noticing the catechol overproduction on CAS agar.

In an attempt to gain physiological data on the organisms' ability to assimilate nutrients Biolog MicroPlates™ were employed. It is worth mentioning that at this stage these plates come in two formats: Those that have a Tetrazolium dye (GP2) to differentiate between positive growth (purple) and negative growth (clear) [Figure 24/Appendix II] and those without (GFN2). The latter relies upon being able to measure growth as a change in turbidity. During this work it was found that Tetrazolium is toxic to many actinomycete cultures and as a result many assays were repeated with the GFN2 plates that were Tetrazolium free.

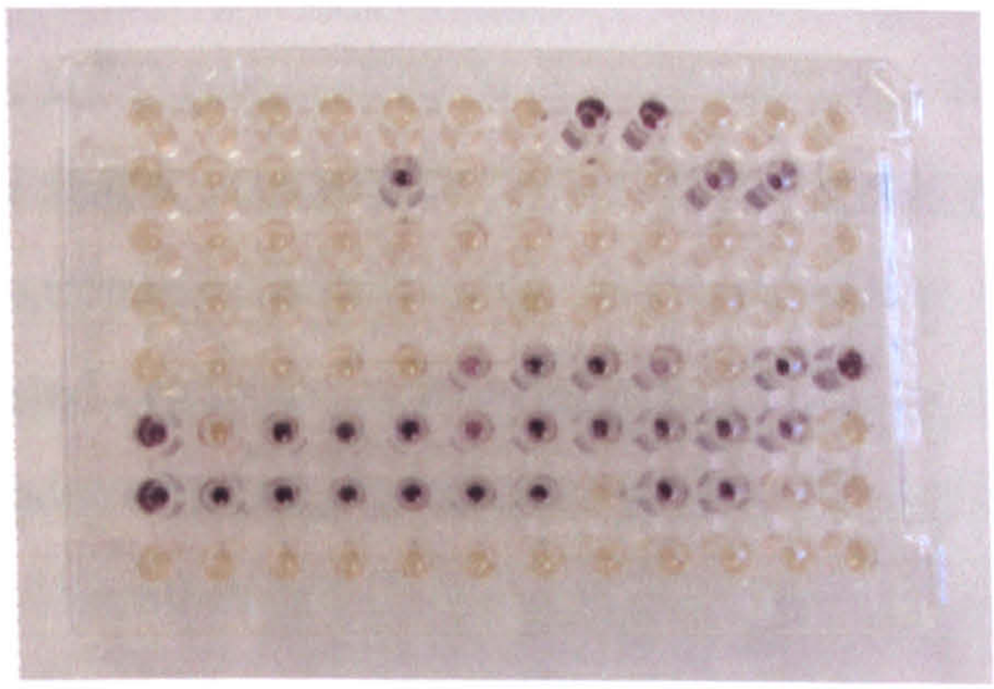


Figure 24: A representation of a GP2 MicroPlate™, Biolog. It demonstrates the nutrient requirements of 29C. Purple dye (tetrazolium) illustrates a positive reaction. The first well (top left) contains water and is used as a control (see Materials and Methods for description).

3. 3. 2. Description of 33D (GenBank accession number EF585406):

It is a Gram positive, aerobic streptomycete species producing brown spirales spores (Figure 25). It grew rapidly on MS, starch-casein, yeast extract-malt extract (YEME) and slightly slower on Muller-Hinton-Fructose (MHF) or Muller-Hinton-Glucose (MHG). It was highly pigmented when grown on YEME and MS; nevertheless it did not sporulate well in the former cultivation media (Figure 25).

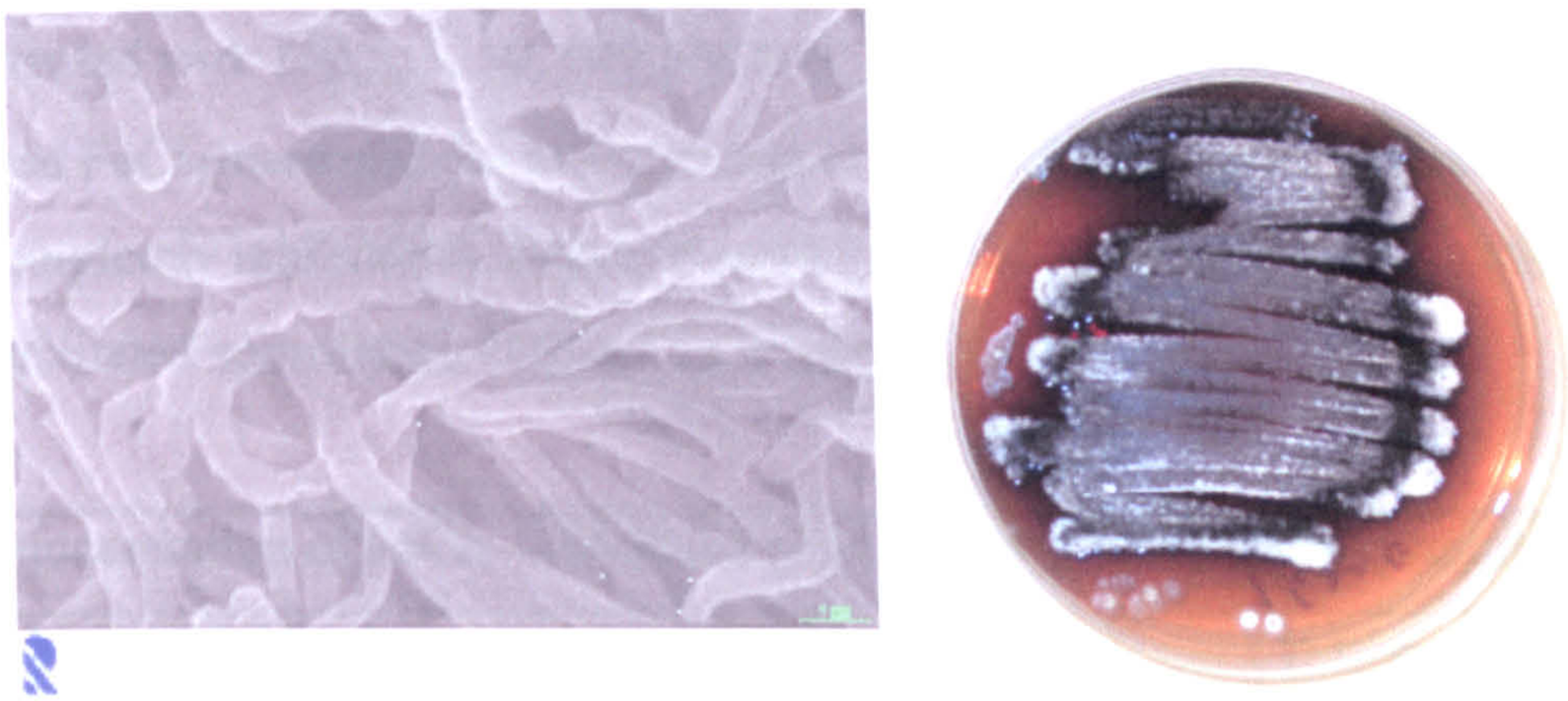


Figure 25: Scanning electron microscopy image of 33D (left) [magnification 10000x] and cultivation on YEME agar, where strong pigmentation is observed (right).

It grew poorly on MS media at pH 5, and appears to be unstable at pH 7. Optimum growth on MS, pH 8 at 30^0 C (Figure 26). It showed antimicrobial activity against *B. subtilis* and was resistant to tetrazolium allowing the application of the GP2 MicroPlate™. It was extremely tolerant to Dip (600μM) [Table 8, page 61-62], suggesting a well-adapted organism in iron-limited environments. It is worth noticing that it overproduced an as yet uncharacterised siderophore on starch-casein media, whereas it synthesised only a small amount of catechol siderophore moieties on MS as evidenced by a yellow halo (Figure 27).

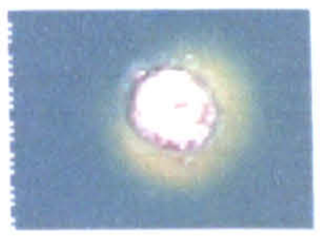


Figure 27: CAS assay demonstrating the production of a small amount of a catecholic compound on MS agar.

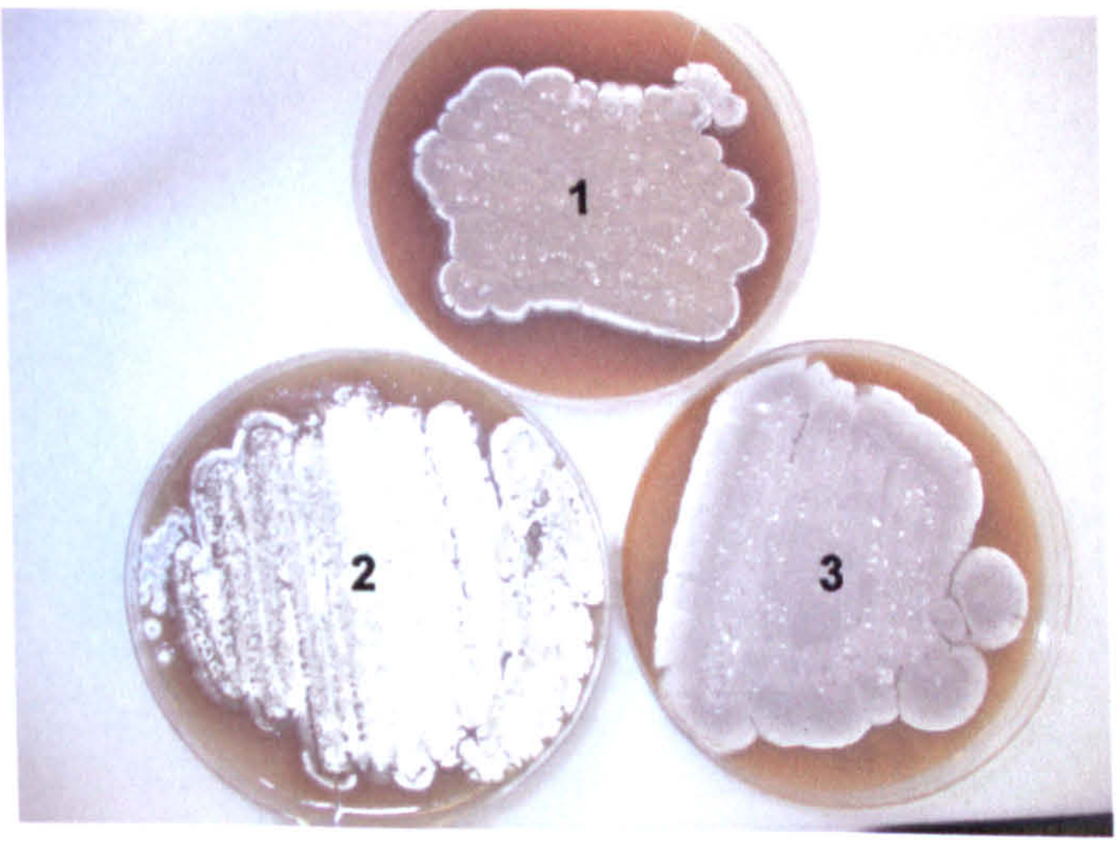


Figure 26: 33D growing on MS agar, pH 6 (1), pH 7 (2) and pH 8 (3). The organism prefers pH 8, where it sporulates much quicker.

The ability of the strain to grow and produce siderophores in the presence of $FeCl_3$ was also examined. It had previously been demonstrated that for *S. olivaceous* addition of 1μM of iron boosted desferrioxamine production by almost 130 %, whereas 10 μM $FeCl_3$ decreased productivity (Meiwes *et al.*, 1990). To test if this phenomenon also applied to strain 33D, the organism was grown on MS agar supplemented with 1, 2.5, 5, 10, 100 μM and 1 mM $FeCl_3$. Medium supplemented with 1 μM $FeCl_3$ increased the biosynthesis of the chelating agent, whereas 1 mM $FeCl_3$ completely inhibited it. However amending the cultivation media with 1 mM of $FeCl_3$ facilitated sporulation (Table 11) [Figures 28-29].

Concentration of $FeCl_3$	Biomass production	Siderophore production
1.00 μM	+	++++
2.50 μM	++	+++
5.00 μM	++	+++
10.0 μM	+++	++
100 μM	+++	+
1.00 mM	++++	-

Table 11: The response of 33D to supplementation of $FeCl_3$ in terms of biomass and siderophore production (as measured by the CAS assay). Maximum production of siderophore or biomass was scored as ++++ whereas the complete absence of siderophore production was scored as -.

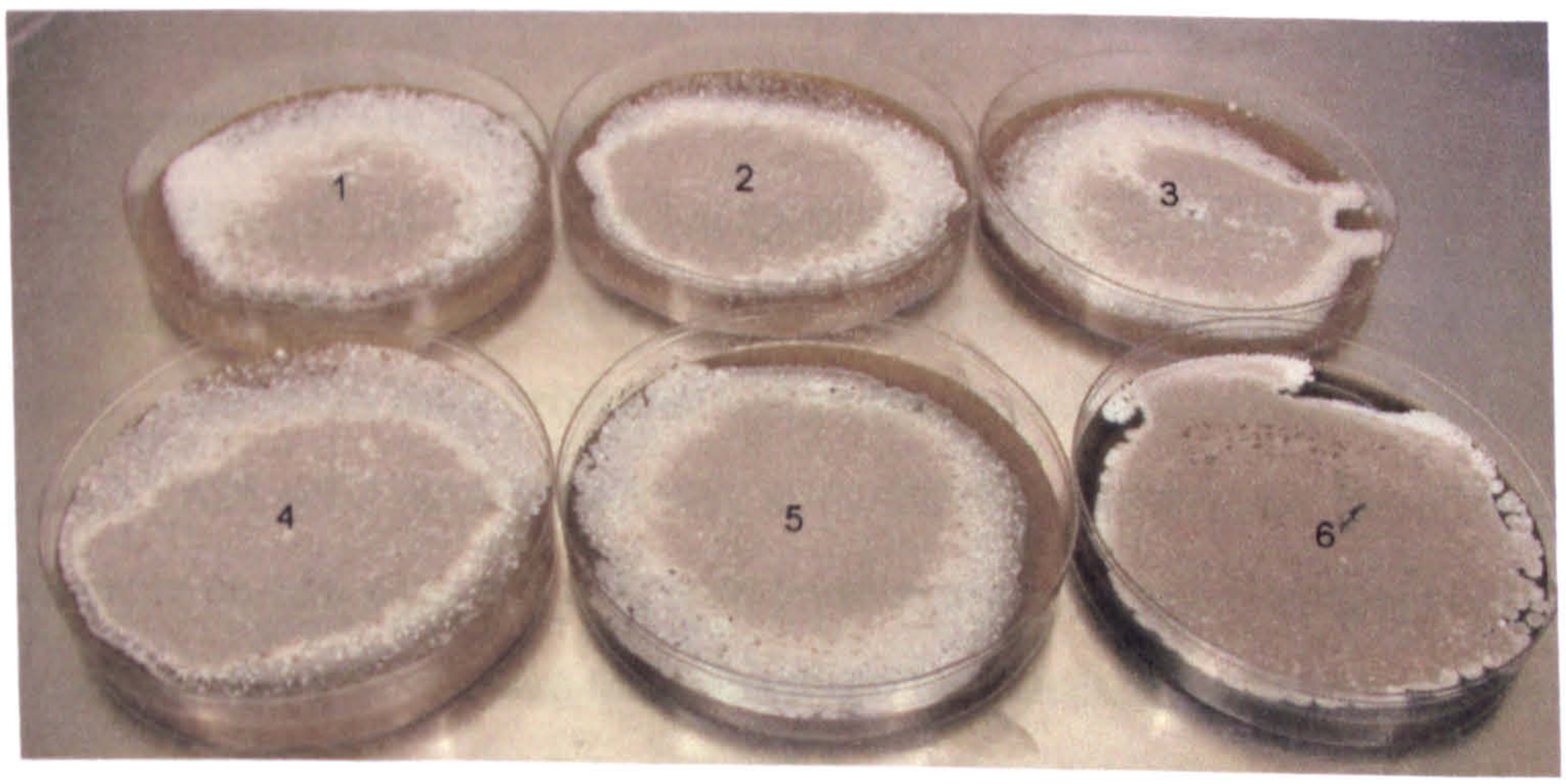


Figure 28: The effect of iron on 33D growing on MS agar. 1: 1 μM $FeCl_3$, 2: 2.5 μM $FeCl_3$, 3: 5 μM $FeCl_3$, 4: 10 μM $FeCl_3$, 5: 100 μM $FeCl_3$ and 6: 1 mM $FeCl_3$.

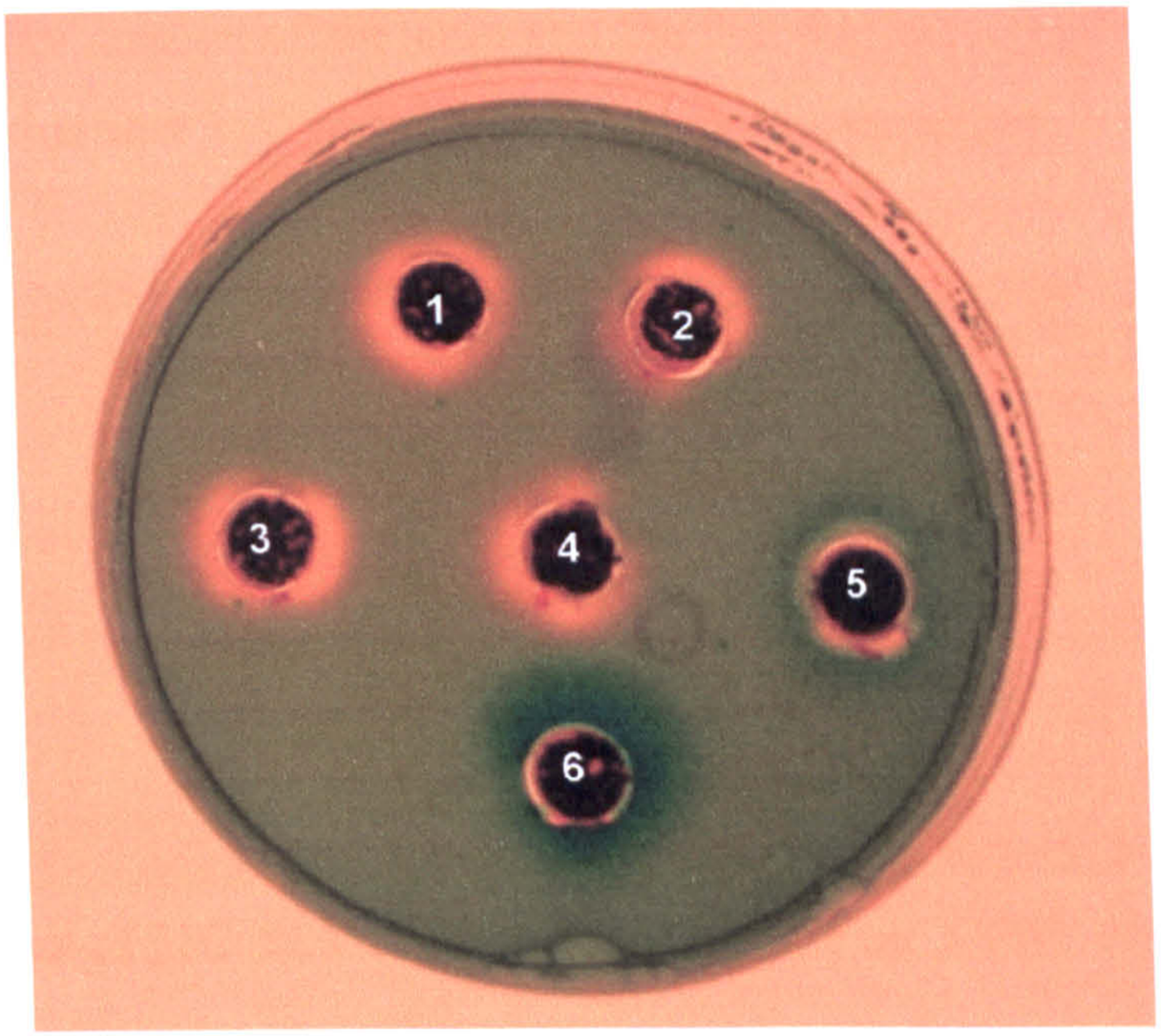


Figure 29: A CAS assay demonstrating the effect of iron on siderophore production of 33D growing on MS agar. 1: 1 μM $FeCl_3$, 2: 2.5 μM $FeCl_3$, 3: 5 μM $FeCl_3$, 4: 10 μM $FeCl_3$, 5: 100 μM $FeCl_3$ and 6: 1 mM $FeCl_3$. Agar plugs (1cm) were removed from MS cultures (7 days old). The blue halo around six is due to the iron transfer for 33D to the CAS agar.

To summarise, 33D was a fast growing organism, a potential siderophore hyper synthesiser and possibly a multiple producer and therefore a candidate for further investigation. However when grown in liquid media it produced large dense pellets (approximately 2.5 mm) with poor mass transfer characteristics. Also because of the tendency towards pelleted growth, this organism was unsuitable for scale-up due to the consequent low number of foci on subculture. Further studies were therefore not conducted on the strain.

3. 3. 3. Descriptions of 31C and 31B:

3. 3. 4. Description of 31C (GenBank accession number EF585405):

31C is a Gram positive, aerobic streptomycete producing rectiflexible white/grey spore chains. This strain only produced substrate mycelia on MHG or MHF agar, whereas on YEME solid media the strain appeared to be extremely unstable (Figure 30). It did grow on MS agar, pH 5-7, with a preference for pH 7 where it only formed aerial mycelia (Figure 31). It was only observed to sporulate on starch casein media with an optimum growth at pH 7.5 at 30^0 C. It exhibited antibiosis against both *B. subtilis* and *C. albicans* but only in media where catechol production was

negligible. The combination of the latter and the fact that the organism grew slowly led as to the decision that 31C was not the ideal candidate either.

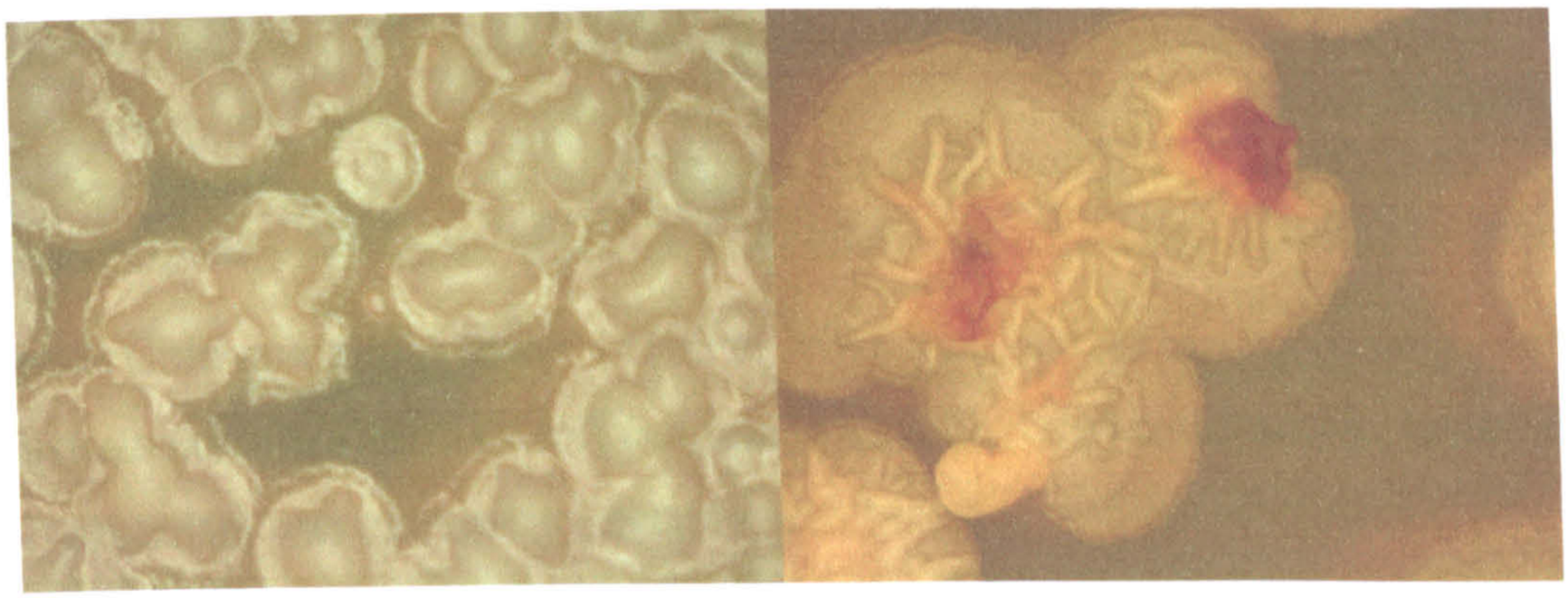


Figure 30: Growth of 31C on MHG or MHF (left) and on YEME (right) solid media.

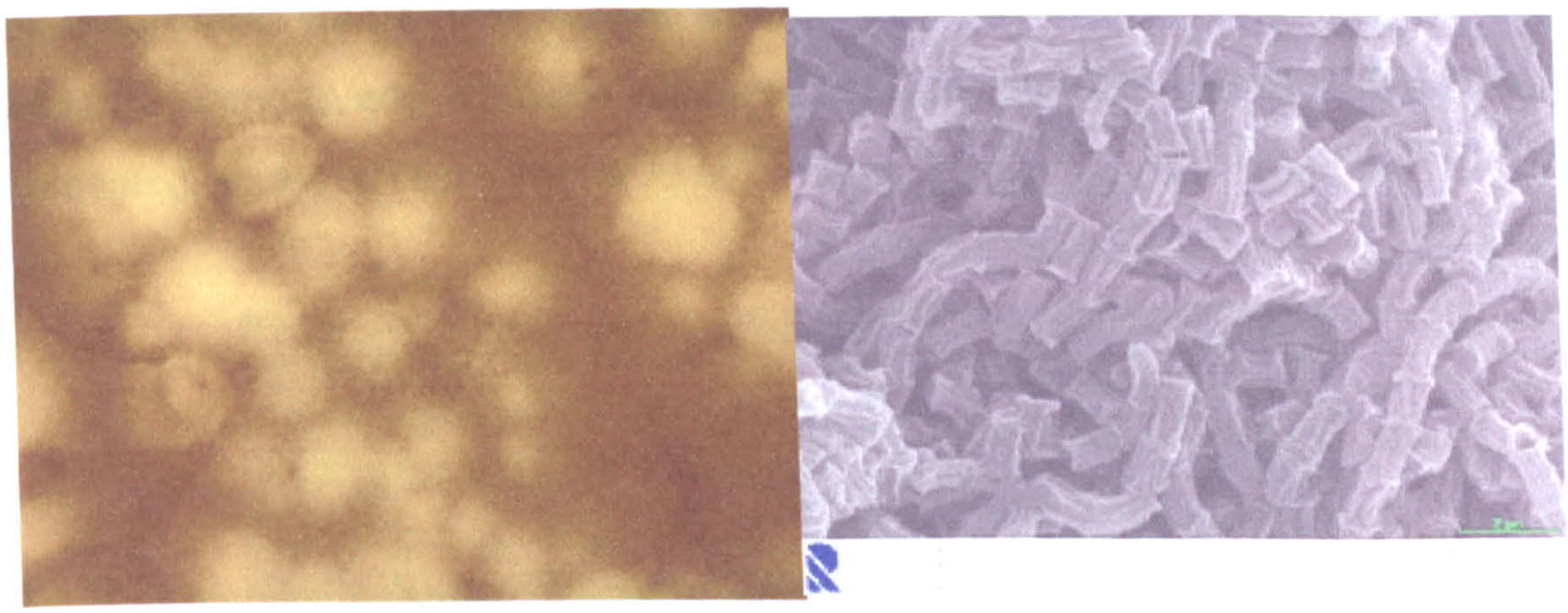


Figure 31: Light microscopy (Magnification 20x) of 31C growing on MS agar, where it only produces aerial mycelia (left). Electron scanning microscopy image of 31B (right) [magnification 8000x].

3. 3. 5. Description of 31B (GenBank accession number EF585404):

This isolate is a Gram positive, aerobic streptomycete producing rectiflexible spores (Figure 31). It was apparent that 31B had relatively fastidious growth requirements compared with many of the other strains being handled at this stage. It also exhibited varied colony formation in our hands (Figure 32). It was also evident that its growth rate was extremely slow with a preference for starch casein media, pH 7.5, at 30^0 C. The description of variable colony morphology is typical on our hands of an organism that is genetically unstable, the result of which is the generation of bold mutants.

Figure 32: Cultivation of 31B on: MHG or MHF agar, where the strain only produced substrate mycelia (left); YEME (middle) and MS (right) solid media, where the organism was extremely unstable, exhibiting varied colony formation.

3. 3. 6. Description of 23F (GenBank accession number EF585403):

This strain is a Gram positive, aerobic actinomycete species, which belongs to the genus *Streptomyces*, and produces extraordinary and abundant spores with an ornamented, spiny, surface (Figure 33).

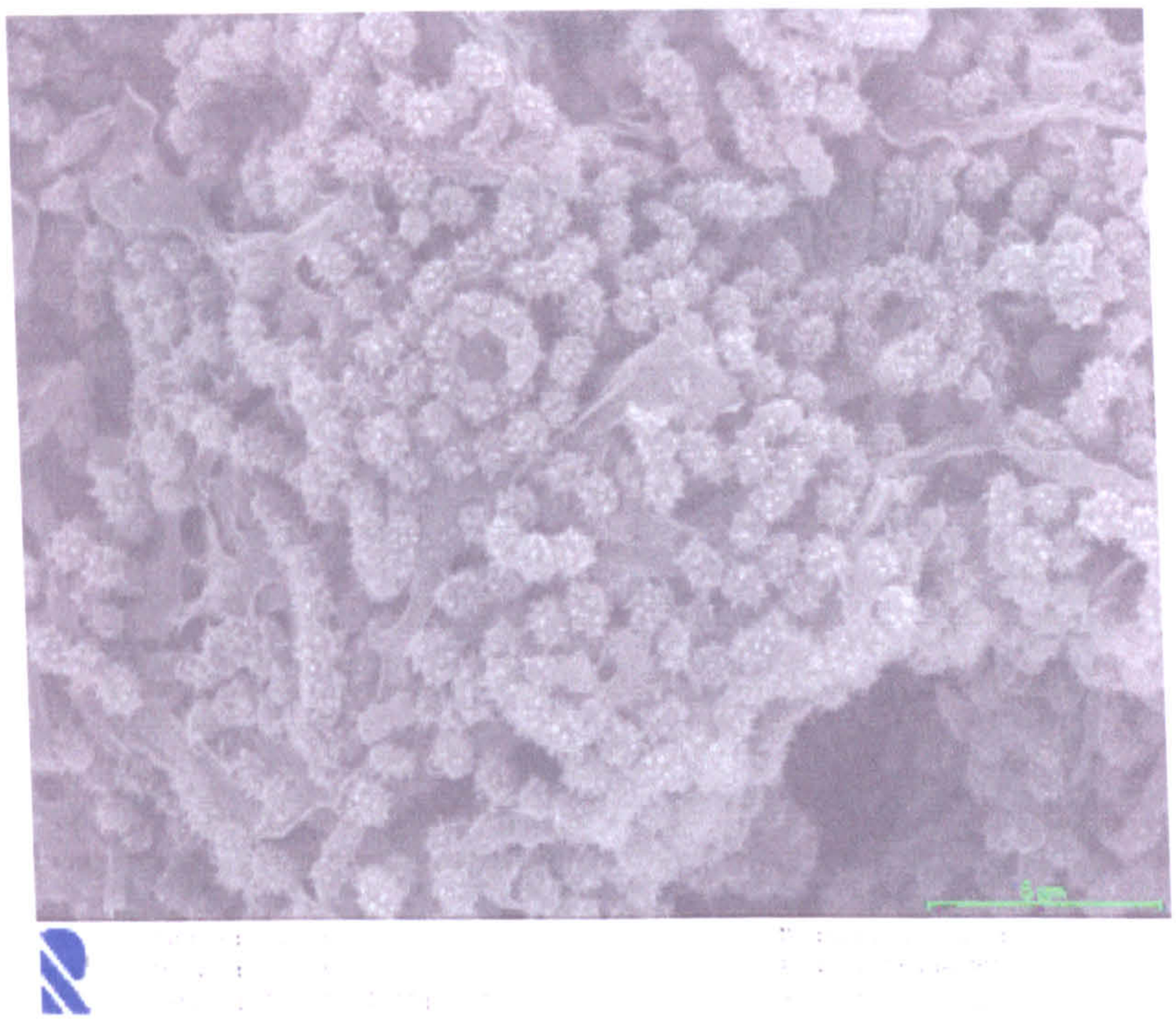


Figure 33: Scanning electron microscopy image (magnification 5000x) of 23F growing on starch-casein agar.

The organism was well adapted to its environment tolerating up to 600 μM Dip (Table 8, page 61-62) and growing in a range of pH (5-8) and temperature (28^0 C- 37^0 C) conditions (Figure 34). It grew quickly on MS agar, nevertheless much slower in starch-casein media taking approximately 10 days to form spores. It did not sporulate

on YEME agar and on MHG or MHF agar. There was also some evidence of phages in the culture seen as sporadic bold patches on the confluent planes (Figure 35). Optimum growth conditions involve cultivation on MS or starch casein, pH 7, at 37^0 C.

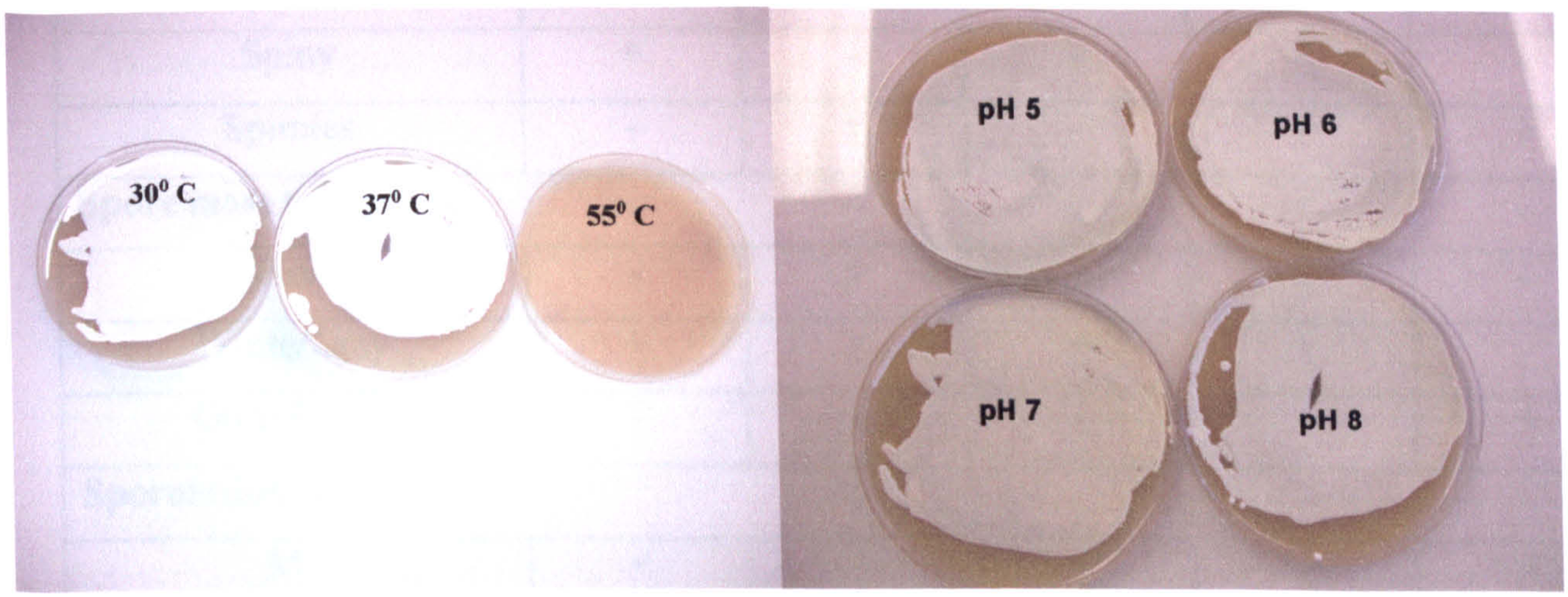


Figure 34: 23F growing on MS agar under different temperature (left) and pH (right) conditions.

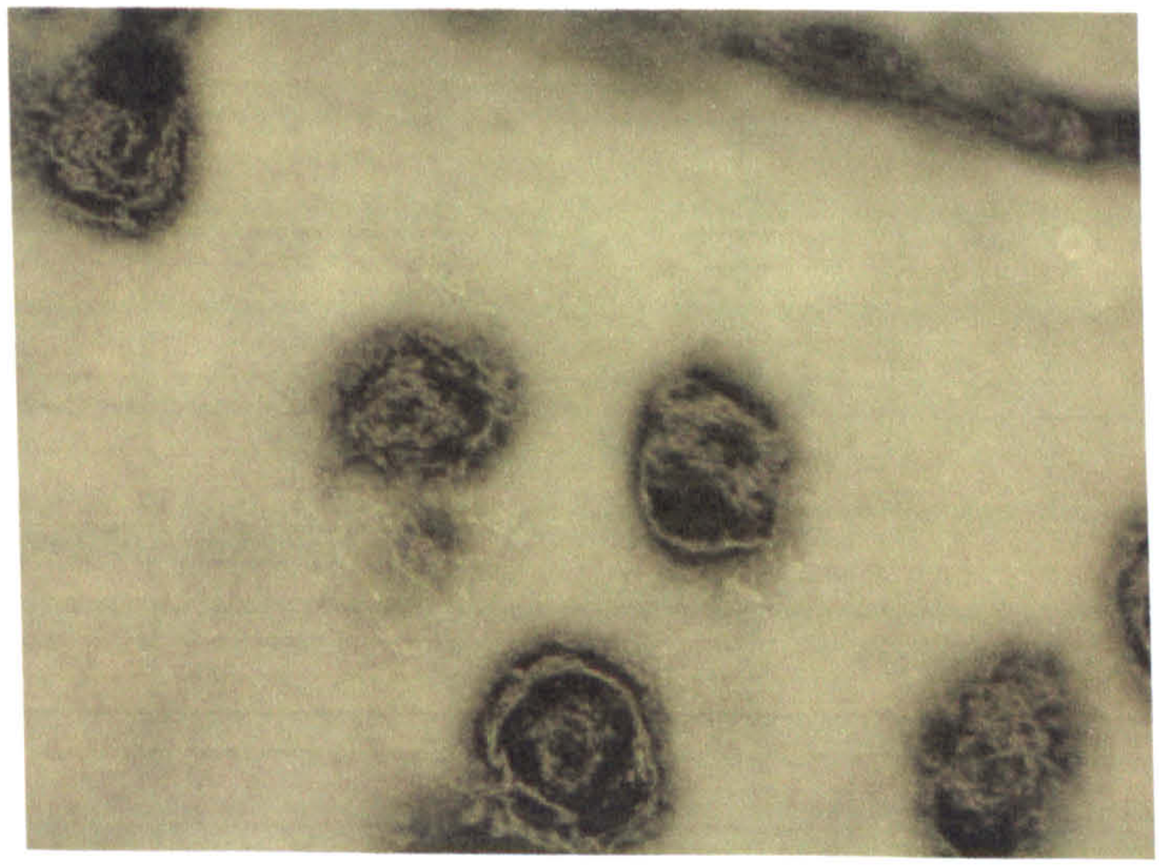


Figure 35: Light microscopy image of 23F growing on MHF or MHG agar, which indicates the possible presence of phages.

Characteristics	Isolates				
	23F	29C	31B	31C	33D
Aerial mycelium:	+	+	+	+	+
Spore chain morphology:					
Rectiflexible	-	-	+	+	-
Spiny	+	-	-	-	-
Spirales	-	+	-	-	+
Spore mass colour:					
Pink	-	+	-	-	-
White/Grey	+	-	+	+	-
Grey/Brown	-	-	-	-	+
Sporulation in:					
MS	+	-	-	-	+
Starch-Casein	+	+	+	+	+
YEME	-	+	-	-	-
MHF, MHG	+	ND	-	-	+
Melanin production:					
Starch-Casein	-	-	-	-	-
MS	-	+	-	+	+
MHF, MHG	+	-	+	-	-
YEME	-	-	+	-	+
Antibiosis against *Bacillus subtilis*:					
MS	-	+	-	-	+
Difco 547 ISP4	-	+	-	+	+
Ammonia limited Difco	-	+	-	+	+
Phosphate limited Difco	-	+	+	+	+
Antibiosis against *Candida albicans*:					
MS	-	+	-	-	-
Difco 547 ISP4	-	+	-	+	-
Ammonia limited Difco	-	+	-	-	-
Phosphate limited Difco	-	+	+	-	-

Resistant to:					
Amoxycillin 2µg	+	-	+	+	+
Amoxycillin/clavulanic acid 3µg	+	+	+	+	+
Ampicillin 25 µg	+	+	-	LG	-
Cefotaxime sodium 5 µg	+	+	+	+	+
Ceftriaxone 30 µg	+	-	-	+	+
Cefuroxime 5 µg	+	-	+	+	+
Cephalexin 30 µg	+	+	+	+	+
Chloramphenicol 50 µg	-	+	+	-	-
Ciprofloxacin 1 µg	+	-	+	+	+
Colistin sulphate 100 µg	-	-	-	-	-
Erythromycin 5 µg	+	-	-	-	-
Gentramicin 10 µg	-	-	-	-	-
Gentramicin 200 µg	-	-	-	-	-
Kanamycin 30 µg	-	-	-	-	-
Levofloxacin 1 µg	+	-	+	+	+
Nalidixic Acid 30 µg	+	+	+	+	+
Netilmicin 10 µg	-	-	-	-	-
Netrilmicin 30 µg	-	-	-	-	-
Nitrofurantoin 50 µg	+	+	+	+	+
Nitrofurantoin 200 µg	+	-	-	+	+
Oxaxillin 1 µg	+	-	+	+	+
Penicillin G 1 unit	+	+	+	+	+
Piperacillin/ tazobactam 85 µg	+	+	-	-	+
Piperacillin/tazobactam 110 µg	+	-	+	+	-
Spectinomycin 25 µg	+	-	-	+	+
Streptomycin 25 µg	-	-	-	-	-
Sulphamethoxazole 25 µg	-	-	ND	ND	ND

Tetracycline 10 µg	-	-	-	+	+
Tetracycline 100 µg	-	-	-	-	-
Trimethoprim 2.5µg	+	+	-	+	+
Vancomycin 5 µg	-	-	LG	-	-
Growth at:					
pH 4	-	-	-	-	-
pH 5	+	-	+	+	+
pH 6	+	ND	+	+	+
pH 7	+	+	+	+	+
pH 8	+	+	+	-	+
pH 9	+	-	-	-	–
28^0 C	+	+	+	+	+
30^0 C	+	+	+	+	+
37^0 C	+	+	+	+	+
55^0 C	-	-	-	-	-
Nutrient utilisation of: **(Measured using the Biolog system)**					
α-Cyclodextrin	+	-	+	-	+
β-Cyclodextrin	+	-	+	+	+
Dextrin	+	-	+	+	+
Glycogen	+	-	+	+	+
Inulin	+	-	+	+	+
Mannan	+	-	-	-	+
Tween 40	+	+	+	+	+
Tween 80	-	+	-	-	+
N-Acetyl-D-Glucosamine	+	-	-	-	+
N-Acetyl-β-D-Mannosamine	+	-	+	-	+
Amygdalin	-	-	-	-	-
L-Arabinose	+	-	+	-	+
D-Arabitol	-	-	+	-	-
Arbutin	+	-	+	-	+
D-Cellobiose	+	-	+	-	+

D-Fructose	+	+	+	-	+
L-Fucose	+	-	+	+	+
D-Galactose	-	-	+	-	+
D-Galacturonic Acid	-	-	+	-	+
Gentiobiose	-	-	+	-	+
D-Gluconic Acid	+	+	+	+	+
α-D-Glucose	+	+	-	-	+
m-Inositol	+	-	+	+	+
α-D-Lactose	-	-	-	-	+
Lactulose	-	-	-	-	+
Maltose	-	-	+	-	-
Maltotriose	-	-	+	-	-
D-Mannitol	-	-	+	-	-
D-Mannose	-	-	-	-	-
D-Melezitose	-	-	+	-	-
D-Melbiose	+	-	+	+	+
α-Methyl-D-Galactoside	-	-	+	-	+
β-Methyl-D-Galactoside	-	-	-	-	-
3-Methyl Glucose	+	-	-	+	+
α-Methyl-D-Glucoside	+	-	+	+	-
β-Methyl-D-Glucoside	-	-	-	+	-
α-Methyl-D-Mannoside	-	-	-	+	+
Palatinose	+	-	+	+	+
D-Psicose	-	-	+	-	-
D-Raffinose	-	-	-	-	-
L-Rahamnose	-	-	+	-	-
D-Ribose	+	-	+	-	+
Salicin	-	-	-	-	+
Sedoheptulosan	-	-	-	-	-
D-Sorbitol	+	-	-	-	-
Stachyose	+	-	+	+	+
Sucrose	+	-	+	-	+
D-Tagatose	+	-	-	-	+
D-Trehalose	-	-	-	-	-

Turanose	+	-	-	+	-
Xylitol	-	-	-	-	-
D-Xylose	-	-	-	-	-
Acetic Acid	+	+	+	-	+
α-Hydroxybutyric Acid	-	+	-	-	-
β-Hydroxybutyric Acid	-	+	+	+	+
γ-Hydroxybutyric Acid	-	+	-	-	+
p-Hydroxy-Phenylacetic Acid	-	-	-	-	-
α-Ketoglutaric Acid	-	+	+	+	+
α-Ketovaleric Acid	+	+	+	+	+
Lactamide	+	-	+	+	+
D-Lactic Acid Methyl Ester	-	-	+	-	+
L-Lactic Acid	-	+	-	-	-
D-Malic Acid	+	+	-	+	+
L-Malic Acid	+	+	-	-	+
Pyruvatic Acid Methyl Ester	+	+	+	+	-
Succinic Acid Mono-methyl Ester	+	+	-	+	+
Propionic Acid	+	+	+	+	+
Pyruvic Acid	+	+	+	+	+
Succinamic Acid	+	+	+	+	+
Succinic Acid	+	+	-	+	+
N-Acetyl-L-Glutamic Acid	+	-	-	-	+
L-Alaminamide	-	+	+	+	+
D-Alanine	-	+	-	+	+
L-Alanine	+	+	-	+	+
L-Alanyl-Glycine	-	+	+	-	-
L-lysine	+	+	+	+	+
L-Asparagine	+	+	+	+	+
L-Glutamic Acid	+	-	-	-	+

Glycil-L-Glutamic Acid	-	+	-	-	-
L-Pyroglutamic Acid	-	-	-	-	-
L-Serine	+	-	+	+	-
Putrescine	+	+	+	+	+
2,3-Butanediol	+	-	-	-	-
Glycerol	-	-	+	-	-
Adenosine	+	-	+	-	+
2'-Deoxy Adenosine	+	-	+	+	+
Inosine	-	-	+	+	+
Thymidine	+	-	+	+	+
Uridine	-	-	+	-	-
Adenosine-5'-Monophosphate	-	-	+	+	-
Thymidine-5'-Monophosphate	-	-	+	+	-
Uridine-5'-Monophosphate	-	-	-	-	-
D-Fructose-6'-Phosphate	+	-	+	+	+
α-D-Glucose-1-Phosphate	-	-	-	-	+
D-Glucose-6'-Phosphate	+	-	-	-	+
D-L-$_{\alpha}$-Glycerol Phosphate	+	-	-	-	+

Table 10: Characteristics of 29C, 33D, 31B, 31C and 23F. LG: Late growth. ND: Not determined.

3. 4. 0. Candidate selection:

One of the reasons that 23F attracted our attention from the early stages as a potential candidate for our studies was the genetic similarities (98 %) with *S. tendae* and *S. thermocarboxydovorans*, which are closely related to *S. coelicolor*, the fully sequenced model of streptomycetes (Figure 36). Gene clusters responsible for desferrioxamine and coelichelin biosynthesis along with iron acquisition transfer mechanisms in *S. coelicolor* have already been identified providing useful information for comparative studies at genome expression level. Therefore we chose 23F for further investigation with a view to maximize biomass and siderophore production in submerged cultures. Further subculture of this organism removed the presence of the phage providing a more robust strain for further propagation. It is worthy of note that the phage may at a latter stage be a useful tool in releasing any biomass bound ions.

3. 5. 0. 23F: A multiple siderophore producer

The strain was grown on both starch-casein and MS solid media with a view to screening siderophore production using the CAS assay. Interestingly both forms of culture gave CAS positive results. It was discovered that 23F produced two different types of chelating agents depending on the nutrient availability in its environment, classifying it as a multiple siderophore producer. When the organism was grown on starch-casein a large orange halo was observed, indicative of hydroxamate biosynthesis. On the other hand it was noticed that the strain produced a smaller amount of a catechol (yellow halo) when grown on rich media (MS). The strain was then further cultured in liquid forms of starch casein and MS media. HPLC analysis revealed the presence of desferrioxamine E in starch casein grown cultures and this was absent from the MS cultivated broths. Desferrioxamine E was confirmed to be present by comparison to authentic standards and by spiking of the sample to confirm retention times.

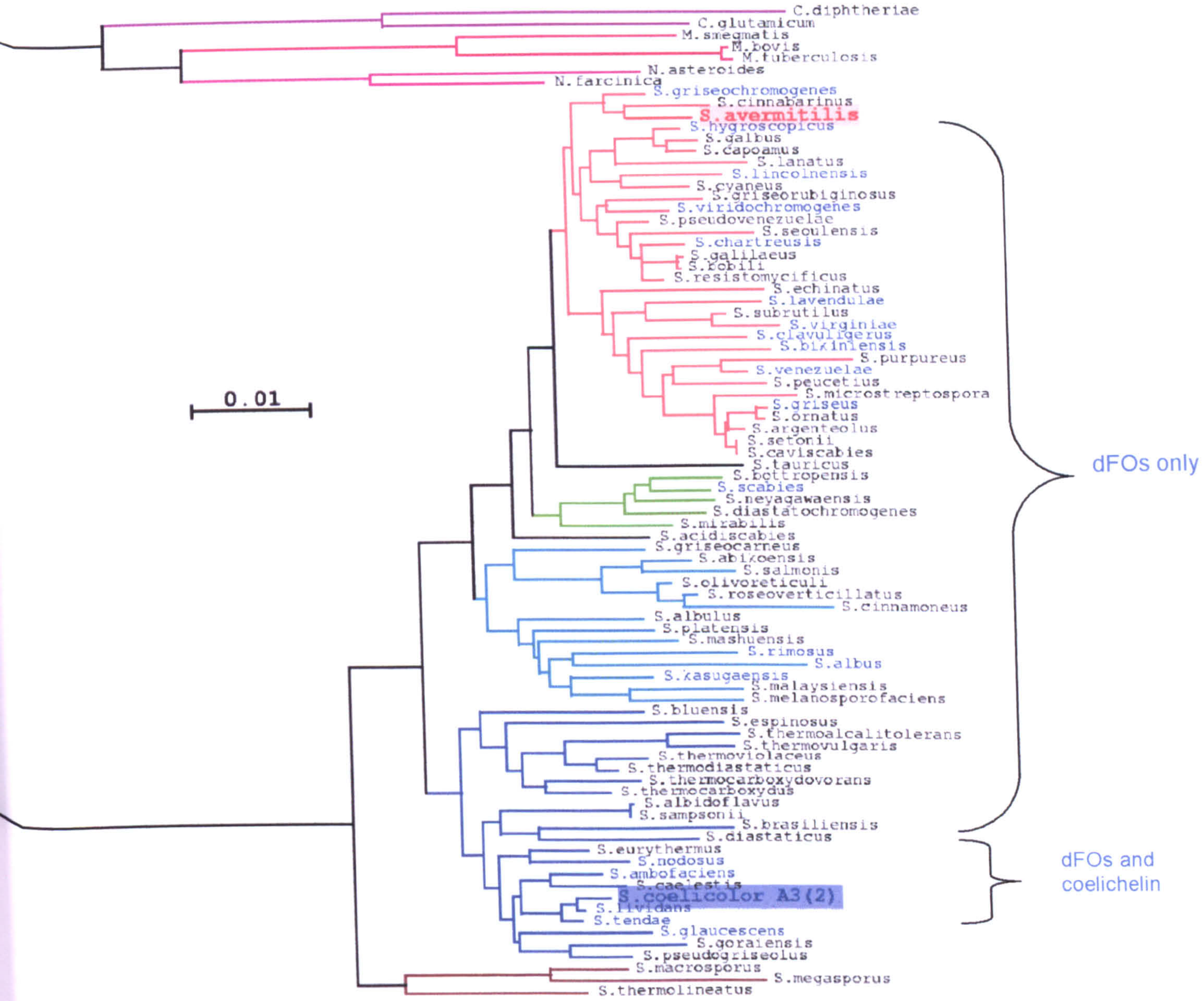


Figure 36: Phylogenetic tree describing the production of desferrioxamines (dFOs) and coelichelin by *Streptomyces* species (Personal communication Dr. P. Barona-Gomez).

3. 6. 0. Desferrioxamine E structure:

From the structure we predicted that desferrioxamine E was made up of three lysine ($C_6H_{14}N_2O_2$) residues and three molecules of succinate ($C_4H_6O_4$); lysine having lost a CO group presumably via a decarboxylation reaction and an OH group through dehydration step; succinate having also lost an OH group too (Figure 37).

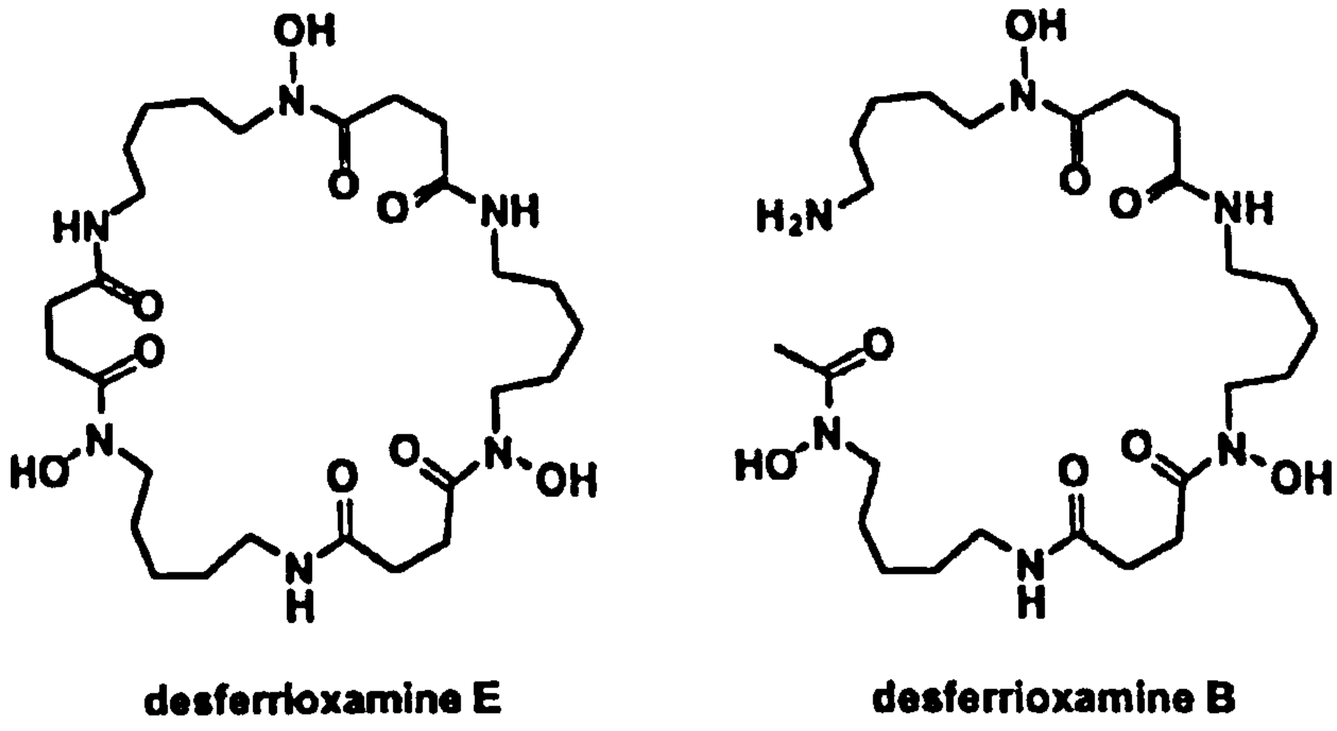


Figure 37: Structure of the desferrioxamines E and B.

Redrawn from Reissbrodt *et al.*, 1990.

Examination of the predicted biochemical pathway for desferrioxamine E biosynthesis accessed from the *S. coelicolor* ScoCyc database (http://scocyc.jic.bbsrc.ac.uk:1555/) confirmed the roles of lysine and succinate as precursors in the metabolic pathway (Figure 38).

During the process L-lysine is decarboxylated by DesA, a pyridoxal-dependent decarboxylase to yield cadaverine, which is further oxidized by DesB, a monooxygenase leading to the formation of N-hydroxycadaverine. Addition of a succinyl group by DesC, a acyl CoA-dependent acyl transferase (acyltransferase) results in the production of N-hydroxy-N-succinylcadaverine (hydroxamic acid). Finally DesD, a desferrioxamine biosynthesis protein catalyzes the final NTP-dependent reaction, which converts 3 molecules of N-hydroxy-N-succinylcadaverine into one molecule of Desferrioxamine E (http://scocyc.jic.bbsrc.ac.uk:1555/).

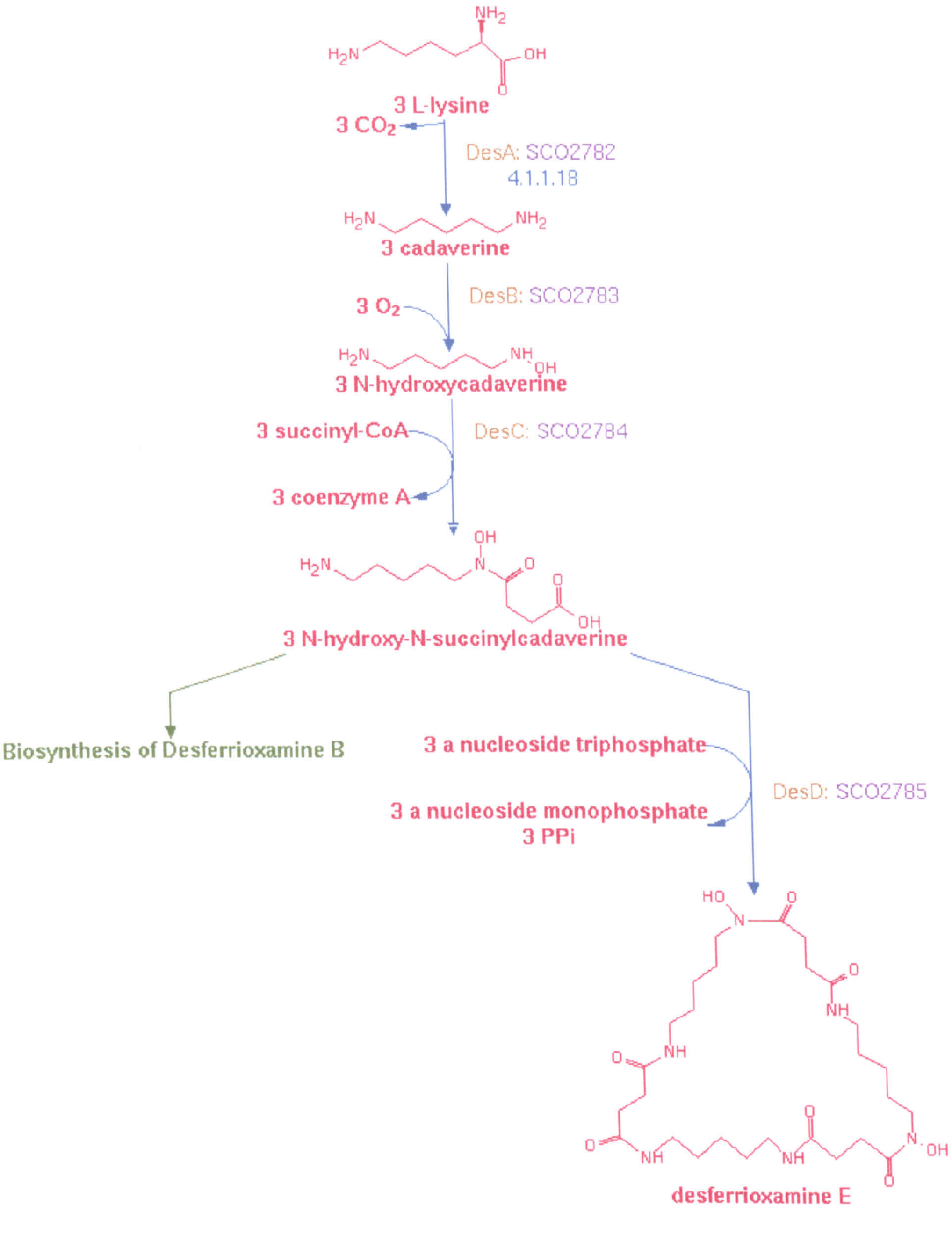


Figure 38: Biosynthetic pathway of desferrioxamine E in *S. coelicolor*. Redrawn from:http://scocyc.jic.bbsrc.ac.uk:1555/SCO/NEWIMAGE?type=PATHWAY&object=PWY1UA-5652.

3. 7. 0. Maximum production of desferrioxamine E production in minimal media:

It has been previously reported that in *S. olivaceous* addition of lysine to the culture media increases the rate of desferrioxamine E formation (Meiwes *et al.*, 1990). The metabolic 'fingerprinting' of the organism by the Biolog microplate had already illustrated that the isolate was able to utilise lysine efficiently (Table 10, page 79). Therefore in order to investigate if this phenomenon occurred in 23F, starch-casein media were amended to include 5 g/L of lysine. In an attempt to further test the organism's limits (in terms of desferrioxamine E production) we cultivated it in the presence of the specific amino acid and different concentrations of the iron chelator, Dip. Initial studies had already demonstrated that Dip enhanced the siderophore production in strain 23F, sending a false signal of iron starvation.

Starch casein is a basal medium that offers minimal nutrients to the organism, resulting in very slow growth rates compared to MS, which is a rich medium. Moreover the presence of Dip stressed the strain further due to sequestration of iron by this compound. Consequently after 10 days the organism was still slowly synthesising the siderophore. In contrast it seemed that the combination of lysine and the chelating agent triggered hydroxamate production reaching its maximum levels after four days (Figures 39 - 40). Remarkably the CAS assay plates could not be scored after 48 hours incubation at 30^0 C because all the available iron on the plate was completely scavenged by 23F. It seemed that lysine supported growth and offered a vital precursor for the biosynthetic pathway. More specifically 5 g/L of lysine increased hydroxamate production by 57 % with a further increase of 74 % in the presence of 200 μM of Dip.

In conclusion, introduction of lysine to the organism increased the rate of siderophore's biosynthesis, which could indicate that 23F can metabolise the amino acid in the formation of the target product. These results enabled us to amend the cultivation media with the view to achieving maximum desferrioxamine yields.

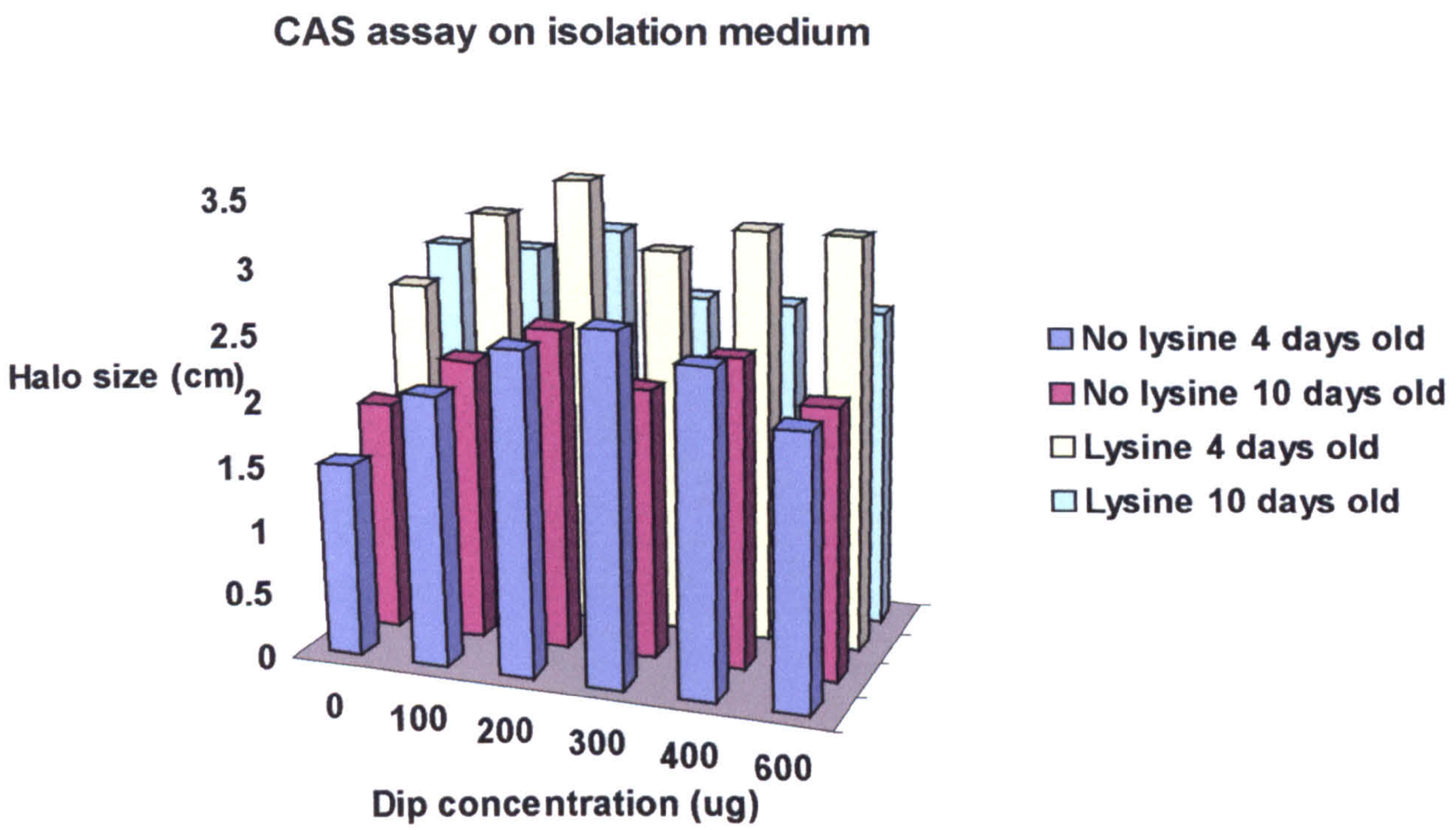


Figure 39: Illustration of desferrioxamine production (CAS assay halo measurements) in the presence of 5 g/L lysine and Dip. The diameter of the well was 1 cm.

3. 8. 0. Effect of various amino acids on desferrioxamine E production:

It is known that amino acids play roles not only as energy sources but as siderophore conjugates too. The previous data based on lysine proved to be so valuable that we decided to screen a variety of amino acids and their combination with a range of Dip concentration (Table 12). A summary of positive effectors are shown in figure 41. Although these amino acids were not obvious direct precursors to the desferrioxamine E biosynthetic pathway, there was evidence from the Biolog system that they can be utilised by the strain. Could transamination reactions occur, that might render these amino acids available to the desferrioxamine pathway?

At day four the organism demonstrated an increase of 57 % in desferrioxamine production when grown in the presence of lysine compared to the control, showing consistency with previous results. In all cases, other than the control and lysine amended media, at day four the addition of Dip lowered the hydroxamate production and affected the organism's biomass levels. Moreover in the majority of instances it appeared that 500 μM Dip stressed the organism, showing weak signs of growth; however it boosted the siderophore production compared to media supplemented with 400 μM Dip. Media amended with 600 μM Dip produced very poor growth; nevertheless significant siderophore production was still measured, indicating that siderophore expression is not simply dependent upon cell biomass.

At day 10, most of the cultures generally demonstrated lower levels of hydroxamate production apart from ornithine and lysine (without supplementation of Dip), serine and the combination of glutamic/aspartic sodium salts and serine; Interestingly some of these exhibited prolific hydroxamate excretion. We conclude that by feeding certain amino acids to the fermentation system the yield of desferrioxamine E can be improved, directing the bioprocess toward the desired product (Table 13).

Amino acid Supplementation	Dip Concentration	Maximum Desferrioxamine E production at:
Lysine	200 μM	Day 4
Ornithine and lysine	-	Day 10
Glutamic/aspartic sodium Salts and serine	100-200 μM	Day 10

Table 13: Details regarding the maximum desferrioxamine E production during a fermentation system.

In an attempt to explain the increase of hydroxamate biosynthesis in the presence of lysine and ornithine it was thought that the organism could have possibly used the synthetic amino acid ornithine ($C_5H_{12}N_2O_2$) converting it to lysine ($C_6H_{14}N_2O_2$) via a decarboxylation reaction. If this step occurred it could have freed more precursor molecules available for the desferrioxamine E biosynthetic pathway.

On the other hand we discovered that ornithine serves as a precursor for the coelichelin biosynthetic pathway, a hydroxamate produced by the phylogenetically related organism to 23 F, *S. coelicolor* (Figure 42) [http://scocyc.jic.bbsrc.ac.uk:1555/SCO/NEW-IMAGE?type=PATHWAY&object=PWY1UA-7603]. The exact mechanism is described in Figure 42. We were unable to conduct coelichelin assays as this compound was unavailable in pure form. At this point it was decided not to further apply the combination of lysine and ornithine with a view to improve desferrioxamine E yield; ornithine could encourage the production of coelichelin-like moieties, which would interfere with the CAS assay, whose detection of the chelating agents is class and not category specific.

The increased expression of desferrioxamine in cultures (10 days old) amended with succinic acid can be explained by the fact that the supplement is an important carrier of activated acetyl groups and plays a vital role in the TCA cycle, where acetyl CoA is produced due to the breakdown of carbohydrates and certain amino acids and the subsequent formation of CO_2 (Table 12).

PAGE
MISSING
IN
ORIGINAL

	Starch casein solid media													
In the	Cultures 4 days old/ CAS- halo diameter (cm)							Cultures 10 days old/ CAS- halo diameter (cm)						
presence of:	No	100 µM	200 µM	300 µM	400 µM	500µ M	600µ M	No	100 µM	200 µM	300 µM	400 µM	500 µM	600 µM
	Dip	Dip	Dip	Dip	Dip	Dip	Dip	Dip	Dip	Dip	Dip	Dip	Dip	Dip
A	3.3	2.6	2.6	2.6	1.6	2.7	1.3	2.6	2.5	2.7	2.7	2.6	2.7	3.0
G	3.2	2.2	2.2	2.0	1.3	2.2	1.1	2.6	3.0	3.0	3.0	3.0	3.0	3.0
S	2.7	1.1	1.1	-	-	1.1	-	3.0	3.0	2.9	3.0	-	2.7	-
GAS	3.3	2.6	2.6	2.4	2.3	2.6	1.9	2.9	3.5	3.4	3.0	3.0	3.5	3.2
O	2.3	1.3	1.1	1.01	1.2	1.7	1.2	1.9	2.2	2.5	2.0	1.4	2.7	1.1
OL	2.2	1.7	1.7	1.5	1.2	2.5	1.5	3.4	2.7	3.0	2.5	3.2	3.0	2.6
Suc.	1.1	1.1	1.05	-	1.2	1.4	1.2	1.4	2.5	2.9	2.0	3.0	2.5	3.0
L	2.6	3.2	3.5	3.0	3.2	ND	3.2	2.8	2.8	3.0	2.5	2.5	ND	2.5
-	1.5	2.1	2.5	2.7	2.5	ND	2.1	1.8	2.2	2.5	2.1	2.4	ND	2.1

Table 12: A list of the CAS assay results conducted in order to screen a variety of amino acids (5 g/L) combined with different concentrations of DIP in terms of hydroxamate production. A: Aspartic sodium salt, G: L-glutamic sodium salt, S: Serine, O: L-ornithine, L: Lysine, Suc: Succinic acid sodium sal ND: Not determined.

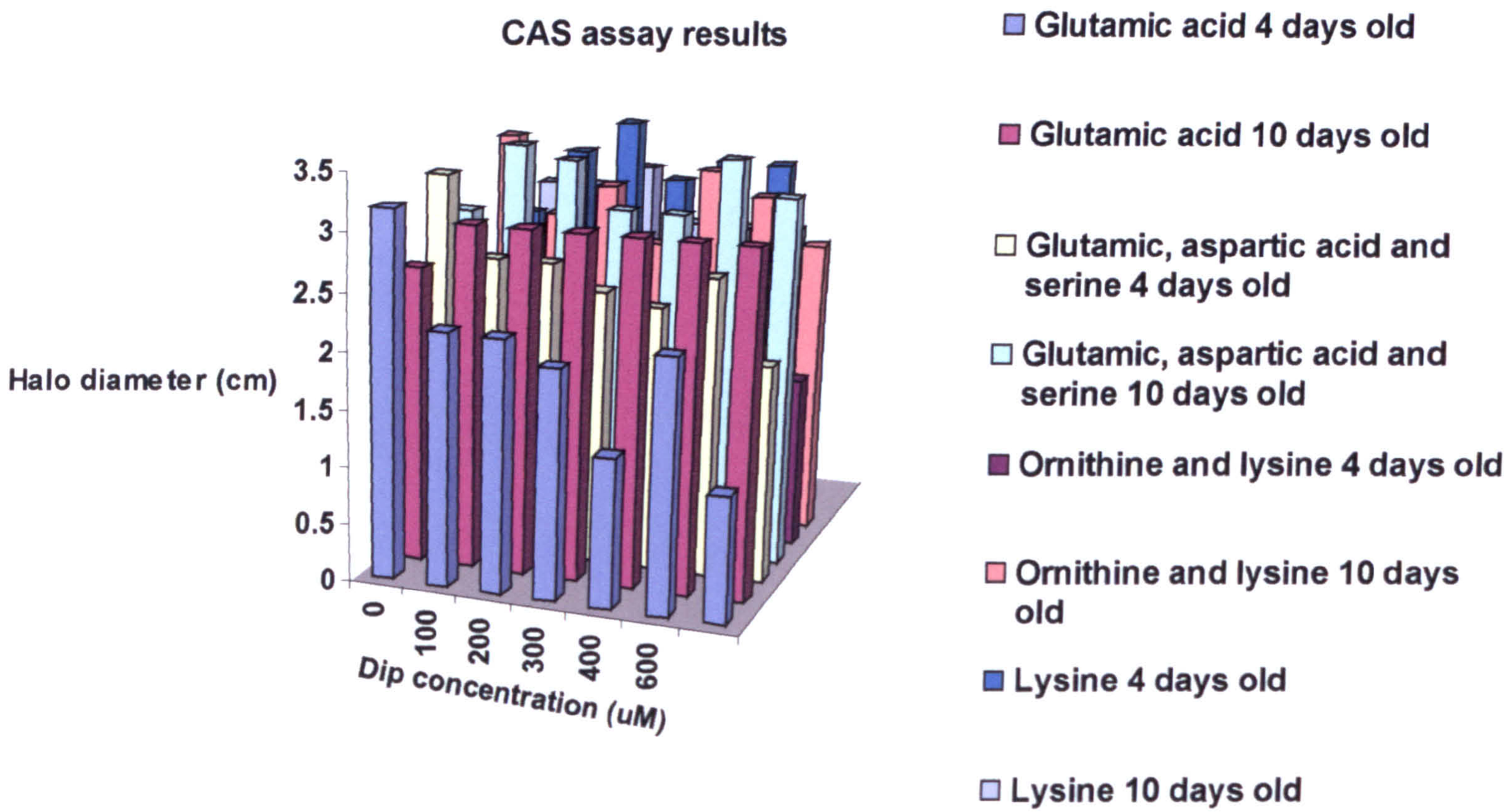


Figure 41: Illustration of amino acids (or combinations) that improved hydroxamate production. It is worth noticing that glutamic sodium salt and lysine demonstrated maximum production at day four, whereas ornithine and the combination of glutamic/aspartic sodium salts and serine had maximum production at day 10.

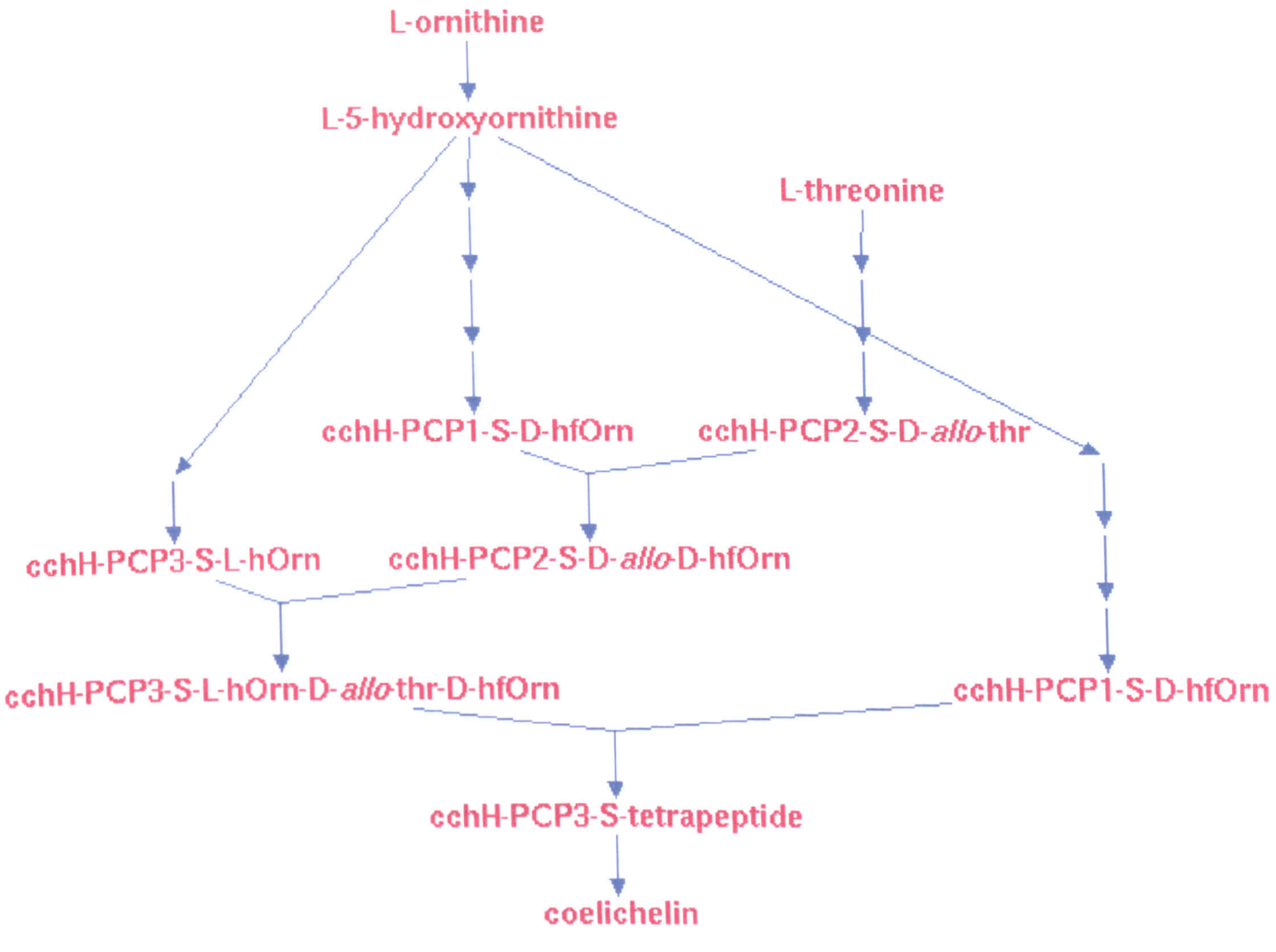


Figure 42: Coelichelin's metabolic pathway in *S. coelicolor*. Redrawn from: http://scocyc.jic.bbsrc.ac.uk:1555/SCO/NEW-IMAGE?type=PATHWAY&object=PWY1UA-7603.

3. 9. 0. Effect of various amino acids on production of catechol based siderophores:

In an attempt to examine the effect of the specific amino acids on catechol biosynthesis, 23F was cultivated on MS media. No differences were observed in terms of siderophore production, indicating that the specific chelating agent might require different precursor(s) for its biosynthesis; nevertheless all amino acids enhanced the strain's growth and sporulation (Data not shown).

Serine appeared to be a particularly promising candidate as it is often found as a precursor of catechol conjugates (Hofte, 1993 and Payne, 1994); however the screen did not demonstrate any relationship between presence of succinate in the medium and catecholic siderophores.

3. 10. 0. Effect of amino acid supplement on the strain's physiology. Is it linked to desferrioxamine E production?

We noticed that the supplementation of amino acids or Dip influenced the strain's physiology. For example the addition of sodium aspartate or the combination of glutamic/aspartic sodium salts and serine suppressed sporulation. Moreover the presence of sodium glutamate delayed sporulation, whereas spore formation was promoted by the addition of ornithine, lysine or their combination. In the light of this it is concluded that siderophore production is not obviously linked to morphological differentiation e.g. aerial mycelium production or sporulation.

3. 11. 0. The effect of amino acid supplementation on cell viability and morphology:

Although it was apparent that the supplements played a role in the strain's morphology, it was not clear if cell viability and pellet formation were either influenced or interlinked by the supplement. In order to establish a robust fermentation system it is essential to understand the relationships between supplementation and morphology. To investigate this MS liquid media were prepared. A number of 250 ml conical flasks containing media (50 ml of MS) and a variety of amino acids supplements were inoculated with 1 ml of a 23F spore stock and incubated in an orbital shaker at 30^0 C for a period of 13 days. All the supplements were added to a final concentration of 5 g/L. Cell viability was determined by fluorescence microscopy using the BacLight^{TM} stain (Cell Viability Kit; Molecular Probes), which colours viable cells green and dead cells red. The cells were examined

at day 8 and 13 days and the differences in terms of viability and morphology were observed (Table 14).

Supplementation of glutamate (G) / aspartate (A), serine (S) or lysine (L) individually demonstrated extensive fragmentation in the culture even when the cultures were old. The fragments were still alive after eight days however after day 13 all cells were dead (Figure 43). In contrast the presence of 5 g/L GAS did not inhibit pellet formation and exhibited the same physiological development as the control (no supplementation). At day 8 when observed both (control and GAS supplemented) cultures contained pellets with a clear staining pattern. Pellet centres were red (dead) while the peripheral hyphae stained green (alive). Surprisingly after 13 days the control culture was mostly dead whereas the amended one (GAS) was still alive (Figure 44).

These data prove that feeding the fermentation with specific amino acids both directs the bioprocess towards the desired biosynthetic pathway and improves the viability of the organism. Therefore we propose the supplementation of 5 g/L GAS and 100-200 μM Dip as a model for a robust fermentation system where the siderophore's yield and strain's performance will be fully controlled and reproducible.

Supplementation of:	MS liquid media			
	Cultures 8 days old		Cultures 13days old	
	Physiology:	Viability	Physiology	Viability
Control. (No supplementation)	Compact pellets	Mostly alive	Compact pellets	Dead
G	Fragmenting	Alive	Fragmenting	Mostly dead
A	Fragmenting	Alive	Fragmenting	Mostly dead
S	Fragmenting	Mostly alive	Fragmenting	Mostly dead
L	Fragmenting	Mostly alive	Fragmenting	Mostly dead
O	Pelleting	Mostly alive	Compact pellets	Mostly dead
GAS	Compact Pellets	Mostly alive	Compact pellets	Mostly alive

Table 14: The effect of amino acid supplementation on the organism's viability as measured by BackLight and physiology. All the concentrations were 5 g/L. A: Aspartic sodium salt, G: L-glutamic sodium salt, S: Serine, O: L-ornithine, L: Lysine.

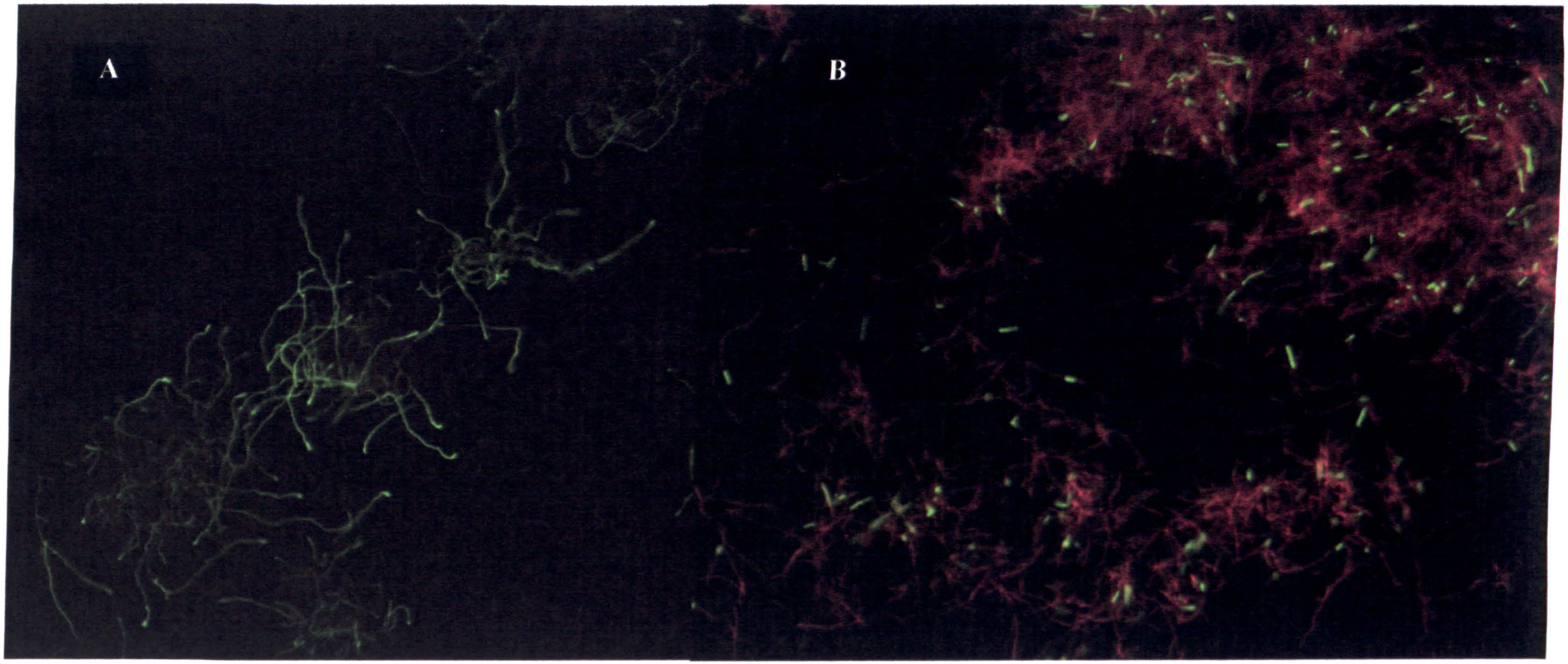


Figure 43: Fluorescent microscopy image of fragmenting cultures at day 8 (A) and day 13 (B) [x 100 magnification]. The live hyphae are stained green, whereas the dead ones are stained red.

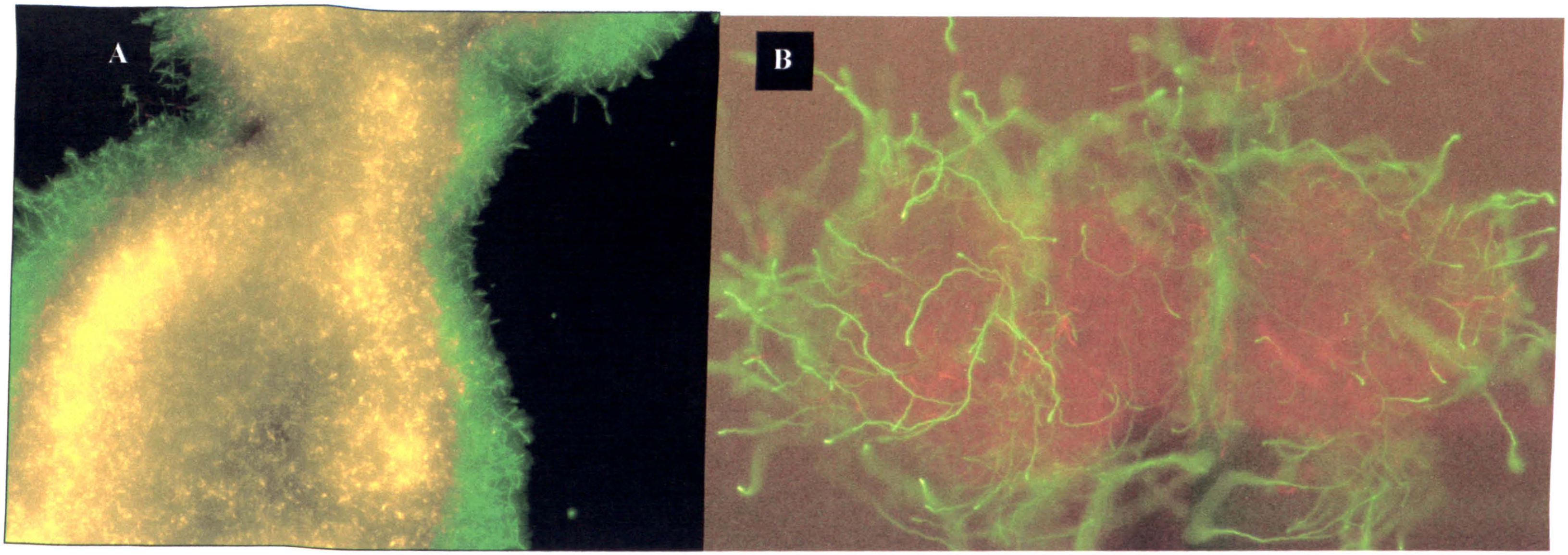


Figure 44: Fluorescent images of 13 days old 23F cultures growing in a control flask (no supplements) [A] and in the presence of 5 g/L GAS (B) [x 630 magnification]. The differences in viability are significant. Image A was collected by deconvolution microscopy. The green areas of the pellets indicate hyphae with intact functioning membranes; the red/ orange areas are those with compromised membranes.

3. 12. 0. The use of fed-batch culture to direct the biosynthesis of hydroxamate siderophore:

The use of MS media provides high biomass and rapid growth of strain 23F compared with the starch casein media. The problem was that MS did not support the production of hydroxamate siderophores. At this point it was decided to attempt to modify the MS medium to facilitate the production of hydroxamate siderophores. Previous work by Meiwes (1990 had described a streptomycetes fermentation to produce a hydroxamate that was fed with sucrose and lysine. Given that the previous section demonstrated the importance of amino acids in the productivity of hydroxamates in this strain and the fact that lysine was a precursor of desferrioxamine E (Figure 38) it was decided to amend MS medium with both lysine and sucrose in a fed batch system similar to that outlined by Meiwes *et al.*, (1990).

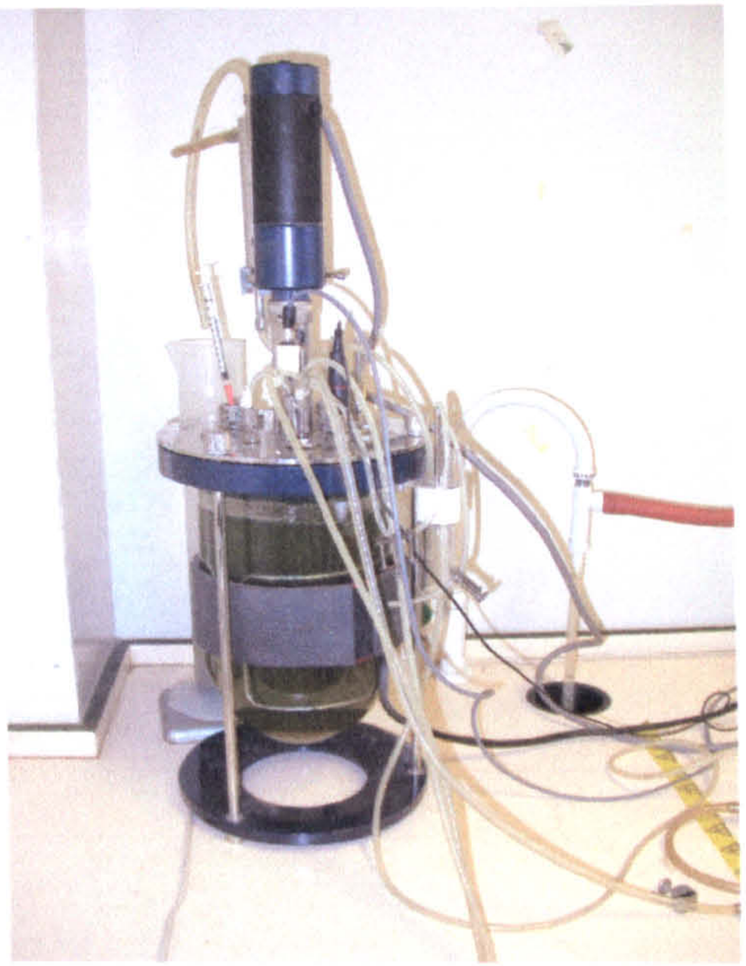


Figure 45: Illustration of the fermentation vessel employed for the cultivation of 23F in liquid media.

A bioreactor containing 4.8 L of MS medium was inoculated with a 24 h old preculture and the fermentation was run as described in Materials and Methods. Samples were taken and analysed for biomass, catechol based siderophores (Arnow's assay) and hydroxamates (Atkin's assay). The culture was grown as a batch system for the first 48 h and at this point was fed with lysine and sucrose. The data (Table 15) show that biomass was produced rapidly over the first 48h, reaching a level of 19.2 g/L at that point. Following the addition of the feed, hydroxamates were detected at 130h and the concentration increased over the remainder of the fermentation up until 178h. This provides us with evidence that we can influence the biosynthesis of siderophore types by addition of biosynthetic pathway precursors.

Time (hours)	PH	Biomass (g/L)	Arnow's assay (OD_{500})	Atkin's assay (OD_{480})
24	6.71	19.2	0.5218	0.0000
48	4.32	17.2	0.2099	0.0000
130	6.29	20.2	0.5337	0.1923
154	7.13	21.9	0.5244	0.239
178	7.36	22.7	0.5889	0.3399

Table 15: Fermentation of strain 23F in MS liquid medium. Arnow's and Atkin's assays were used to measure catechol and hydroxamate biosynthesis respectively. Biomass was estimated by dry weight.

To further demonstrate this, a second fermentation was conducted with the time of feeding set at a later point post inoculation (112 h) [Figure 46]. This was intended to demonstrate whether or not under these conditions hydroxamate was produced as a result of the feeding 'perse' or that simply hydroxamate production was switched on late in a batch fermentation. Following the feed the hydroxamates were detected and biosynthesis continued until the end of the fermentation (178h). The data were also verified by TLC, suggesting further the presence of desferrioxamine E.

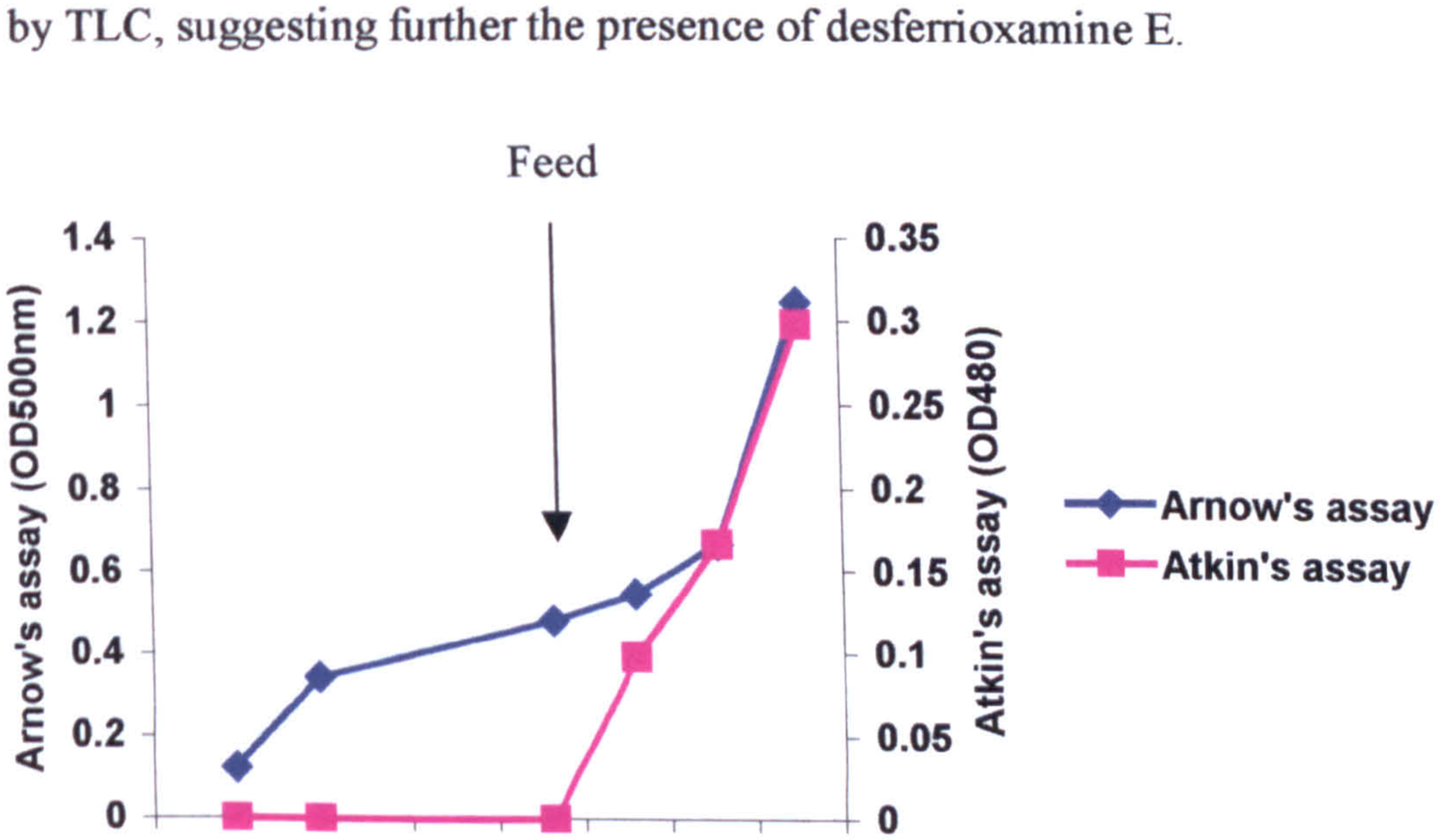


Figure 46: Illustration of siderophore production of 23F during feeding fed batch fermentation. Arnow's and Atkin's assays were used for catechol and hydroxamate production respectively.

Furthermore the fermentation broth was highly viscous consisting of diffuse pellets; demonstrating properties favoured for immobilisation, which makes this system suitable for bioremediation studies (Figure 47).

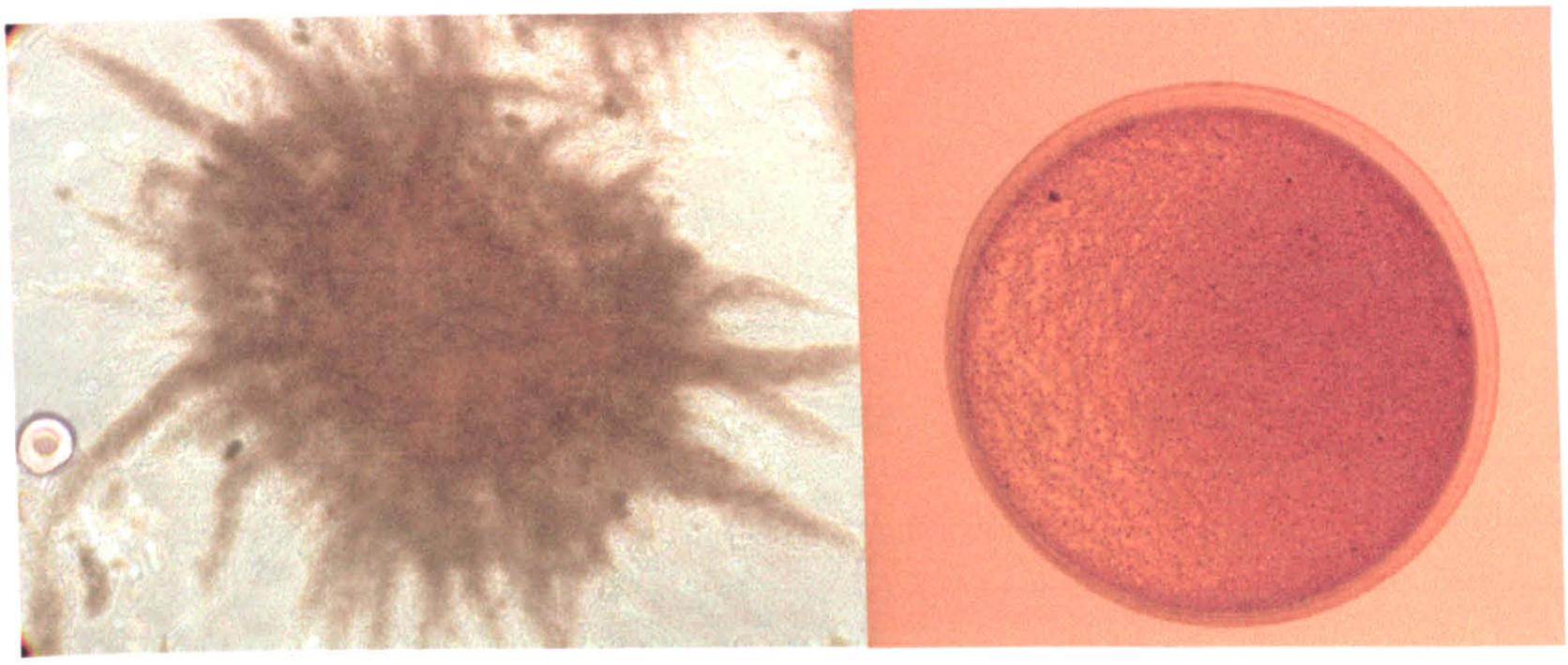


Figure 47: A pellet of 23F grown in a fed-batch culture (left) [Magnification 40x] and highly viscous broth harvested at the end of fermentation (right).

In order to verify these data a small-scale experiment was designed. Three 250 ml conical flasks containing 50 ml of liquid MS were inoculated with 500 µl of 23F spore stock. Supplementation of 5 g/L lysine was performed in two of them whereas the third remained as a control. One of the amended cultures was further 'fed' with 1 g/L of lysine after 48 hours. All of the flasks were incubated in an orbital shaker, 250 rpm, at 30^0 C for 12 days.

At day two HPLC analysis confirmed the presence of desferrioxamine E in the amended media whereas it was clear that no desferrioxamine was produced in the basal media. These results consistently verified strain 23F's status as a multiple siderophore producer depending on the nutrient availability in its environment and also demonstrated how careful media design techniques can allow the bioprocess to be directed towards a desired product.

It is worth mentioning that the hydroxamate production was estimated around 41 µg/ml at day two with a decrease to 0.25 µg/ml at day seven and minor traces at day twelve (Table 16). These are the first data indicating that when the strain consumed all its energy sources, it possibly degraded its own chelating agents to gain vital nutrients, such as amino acids. A similar mechanism has been suggested for the

degradation of desferrioxamine B to monohydroxamates by certain Rhizobia species (Pierwola *et al.*, 2004).

Time (Days)	Desferrioxamine E production (µg / ml)
2	41
7	0.25
10	0.05
12	0.01

Table 16: Desferrioxamine E production in MS liquid medium (measured by HPLC).

Moreover the culture supplied with further lysine (1 g/L) demonstrated a desferrioxamine E production of 63 µg/ml at day seven, suggesting that the organism utilised the extra amount of the amino acid for growth purposes, which subsequently led to further siderophore biosynthesis. Indeed when cell mass was measured this culture demonstrated the highest biomass levels, 7.5 g/L, compared to 4.4 g/L (addition of 5 g/L of lysine) and 3.5 g/L (control). These results suggest that the concentration of the supplements provided to the organism and the timing of the feed have to be optimised in order to make sure that the nutrients provided are directly involved in the siderophore biosynthetic pathway and not utilised from the organism for growth.

3. 13. 0. The role of iron availability on siderophore production:

In order to further understand and control siderophore production the relationship between iron availability and the biosynthesis of the chelating agents had to be investigated. Therefore the minimum amount of iron needed to suppress siderophore production had to be determined in both basal and rich media. Consequently the ability of 23F to synthesise siderophores in the presence of a range of $FeCl_3$ concentrations, 0.2 - 500 µM, was tested.

Desferrioxamine E production was inhibited in starch casein media containing iron even as low as 0.2 µM (data not shown), suggesting that iron's function was to co-repress the pathway possibly by binding to the DmdR or DtxR families. In conclusion it seemed that the hydroxamate production was extremely dependent on iron

deficiency conditions. Similar mechanisms have been observed in closely related species, such as *M. tuberculosis* and *S. coelicolor*.

In contrast the organism was still synthesising the catecholic compound in MS media containing up to 100 μM $FeCl_3$. These data suggested that the strain possessed two independently regulated systems for iron uptake, which are related to the metal availability in the environment (Figure 48).

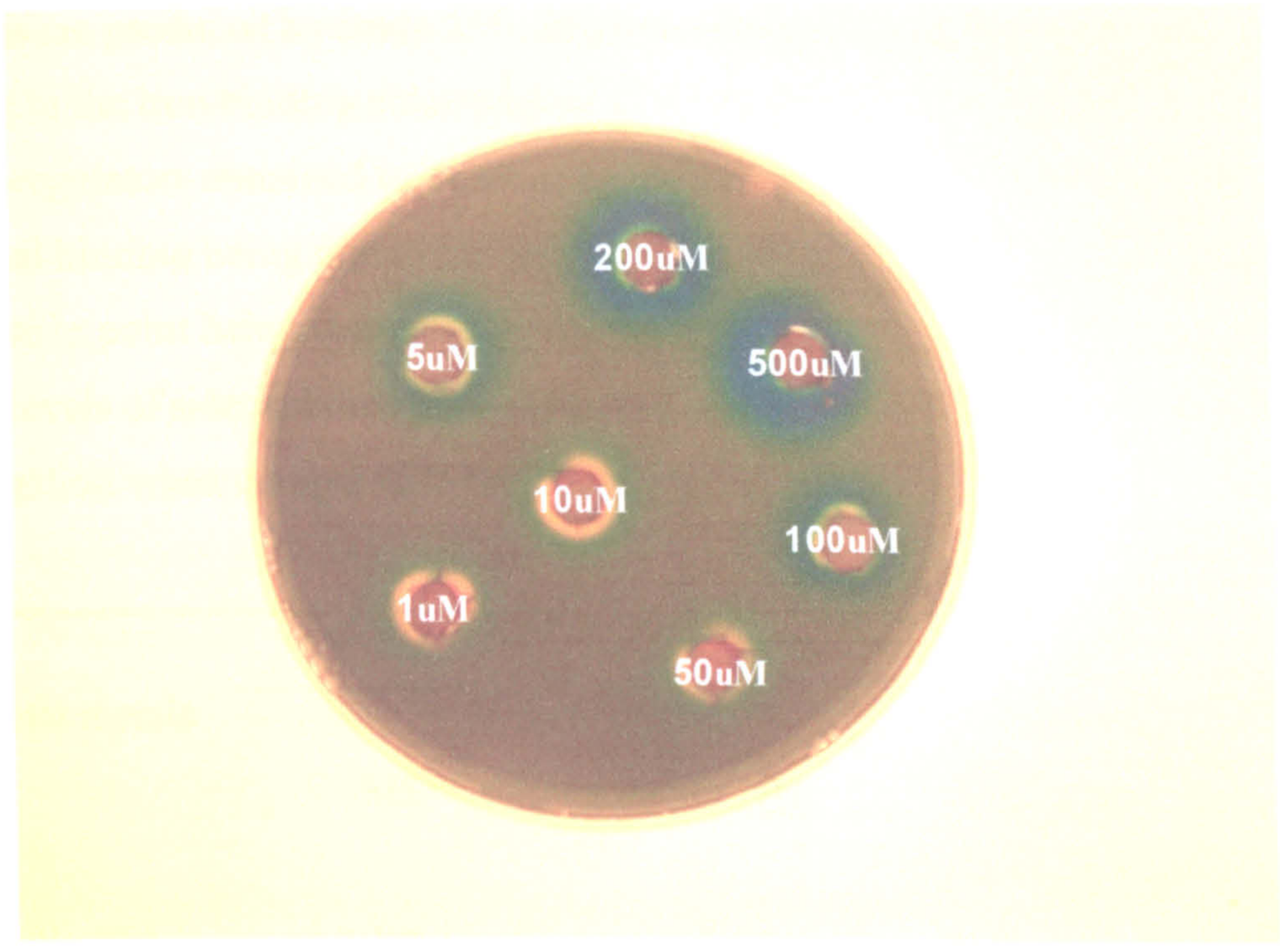


Figure 48: CAS assay demonstrating that 23F still produces a catechol when grown in the presence of 100 μM $FeCl_3$ as seen as a yellow halo.

3. 14. 0. Siderophore-binding metals?

A number of modified CAS assays were developed in order to investigate if the siderophores produced by strain 23F could bind to other metal ions. If other ions could be captured by the siderophores then this could have important implications in the use of this organism for bioremediation of metal contaminated sites. Solid media including MS, starch casein and starch casein supplemented with 5 g/L of lysine were inoculated with the organism and incubated at 30^0 C for four days. MS grown cultures would produce catechol siderophores while starch casein grown cultures would produce hydroxamates. CAS assay plates were formulated such that the ferric ion was replaced with the specific metal ion under investigation. Plugs from the four days old cultures were removed and placed on the assay plates.

After 24 h of incubation marked zones of clearing/decolourisation around the plugs on the CAS assay plates could be seen in the majority of cases (Table 17). Plugs from starch casein based media (hydroxamate producing) gave the greatest size zones indicating that siderophore binding of metal ions other than iron was occurring. Only chromium III did not show a positive CAS assay. Plugs from MS agar plates showed small zones of decolourisation in the assay with the exception of copper II, which gave rise to a much larger halo. This result suggests that general metal binding moieties are produced by strain 23F. Whether this phenomenon is due to non-specific binding to the iron binding siderophores or to the presence of possible metalloregulators remained to be elucidated. Some indirect evidence would point to the metal binding being due to the siderophores normally expressed by this organism. The case in point being that amended casein starch media that we know to express higher levels of siderophores gave greater zone sizes for each of the metals under investigation when compared to basal media.

Metal metals	Halos (cm)		
	MS	Starch casein	Starch casein supplemented with ornithine and lysine (5g/l)
Fe III	1.2	1.9	3.5
Mn II	1.1	3.0	3.6
Co II	1.1	2.6	4.1
Cd II	1.1	3.1	4.1
Ni III	1.1	3.1	4.1
Al III	1.1	1.6	2.6
Li I	1.1	3.1	4.0
Cu II	3.0	2.5	4.0
Cr III	-	-	-
As II	1.1	1.1	ND
Zn II	1.1	1.7	2.8
Mg II	1.1	1.8	3.1

Table 17: CAS assay results describing the siderophores ability to bind a variety of heavy metals. It is worth noticing that catechol (MS) exhibited high affinity for copper. The diameter of the well was 1cm.

In general the catechol (produced in MS) demonstrated a similar chelating behaviour to this towards $FeCl_3$ by forming very strong metal complexes (usually small halos). However its high affinity for copper II was not expected. The results were visible on the plates after 10 minutes. The unusually big halo on the CAS assay probably indicates that the agent scavenges more copper from its environment than iron. This may suggest the presence of a copper binding regulator (Cur?) in the strain's genome. Moreover considering that siderophores are mainly iron- binding compounds, the relationship between iron and metal transport systems warrants further study.

3. 15. 0. Inducer/Repressor of siderophores' biosynthetic pathway:

In order to confirm if metal binding activity was due to siderophore production the modified (including the specific metal) and $FeCl_3$ CAS assays were performed keeping constant the metal concentrations in both tests. The strain was grown for ten days on starch casein solid media and the screen was performed twice (days: two and ten). To further investigate if the ions function as siderophore repressors different concentrations (500 μM- 0.1 μM) of the above-mentioned metals were supplemented (Table 18).

In general (within experimental error) at day four the halo size of the $FeCl_3$ CAS assay was matching the one from the modified CAS test with an exception of copper! Copper immediately gained our attention since iron and copper share a long lasting historical relationship. These results also support early data indicating the possible presence of copper binding regulators in 23F's genome. The experiment was also repeated at day ten demonstrating similar outcomes with smaller halo sizes as was expected.

Not surprisingly iron was found to be the only metal ion repressing hydroxamate biosynthesis. Where growth was observed none of the other metals, in any concentrations employed, inhibited the chelating agent's formation.

At this stage it was not clear if the metals were inducing the siderophore biosynthetic pathway or the absence of iron (a well defined repressor) was relieving repression. In order to clarify the interaction of ions and hydroxamate production the strain was cultivated on minimal media (starch casein) including a combination of the specific ion (in different concentrations) and iron (Table 19).

It was obvious that iron served as the sole repressor of the hydroxamate production (starch casein) as in the presence of the specific metal no hydroxamate was produced. Moreover any other metals trialed failed to induce the siderophore biosynthesis and no competition between the ion combinations was observed.

Supplementation of Fe (III) and Cu (II) into the growth media appeared to repress the ability to bind Fe as evidenced by the CAS assay; however Cu (II) was seen to be bound in the modified CAS assay for this sample. This is the only example where iron was not found to repress a metal ion binding 'capacity'. This outcome may indicate the presence of an independent regulatory system where the ions' (Cu, Fe) acquisition could possibly be controlled by the same mechanism that operates in an unassociated manner depending on the environmental conditions. This hypothesis is in agreement with the contingency theory previously published in 2003 (Challis & Hopwood, 2003).

Another explanation could be the presence of two co-regulators (Cur and Fur?) or the existence of two DtxRs; *S. coelicolor* possess DmdR1 and DmdR2 with different metal binging affinities (Flores & Martin 2004 and Flores *et al.*, 2003).

In order to investigate if iron was the only repressor of the catecholic biosynthetic pathway the experiment was repeated using rich media (MS) (Table 20). The catechol moiety was present in all cultures even in the presence of iron (data not included) and surprisingly the halo size of both CAS assays (iron and modified) was not affected by the different concentrations of ions. This might indicate that catechols, although strong chelating agents, possess certain capacity in terms of ion acquisition.

It is worth mentioning that in high concentrations (500 μM) of Zinc and Manganese a hydroxamate appears to be produced, evident as an orange halo on Fe CAS. It is known that high concentrations of manganese mimic Fe (Escolar *et al.*, 1999). Moreover in rich (MS) media production of catecholic siderophore was not affected by the presence of iron; therefore it does not lead to the repression of the iron uptake system. However why the strain switched from the production of catechol to hydroxamate's is not understood.

Starch casein solid media																			
Metals	Modified CAS (Halo size, cm)									$FeCl_3$ CAS (Halo size, cm)								
	Metal Concentrations (μM)									Metal Concentrations (μM)								
	500	200	100	50	10	5	1	0.5	0.1	500	200	100	50	10	5	1	0.5	0.1
Mn II Growth	1.9	2.2	+	+	2.5	+	2	+	+	2	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Co II Growth	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	~	~	~	~	-	-	-	-	~	~	~	~	-	-
Cd II Growth	-	-	-	-	+	+	+	+	+	-	-	-	-	+	+	+	+	+
	-	-	-	-	Max?	+	+	+	+	-	-	-	-	Max?	+	+	+	+
Ni III Growth	-	-	-	-	-	2.5	2.5	2.5	2.5	-	-	-	-	-	2.5	2.5	2.5	2.5
	-	-	~	+	Max?	+	+	+	Max?	-	-	~	+	Max?	+	+	+	Max?
Al III Growth	-	-	-	-	-	ND	+	+	+	-	-	-	-	-	+	+	+	+
	~	~	+	+	+	+	+	+	+	~	~	+	+	+	+	+	+	+
Li I Growth	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cu II Growth	-	-	-	-	2	2	1.9	2.5	2.3	-	-	-	-	1.2	1.2	1.2	1.3	1.3
	-	-	-	-	+	+	+	+	+	-	-	-	-	+	+	+	+	+
Zn II Growth	-	1.7	1.7	1.7	1.8	2	1.7	1.7	1.7	-	1.7	1.7	1.7	1.8	2	1.7	1.7	1.7
	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+
Mg II Growth	-	+	ND	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+
	~	+	+	+	+	+	+	+	+	~	+	+	+	+	+	+	+	+

Table 18: $FeCl_3$ and modified CAS assays of 23F growing in the present of 500-0.1μM of various metals. – and + indicate the absence and presence (minor) of a halo respectively. ~ indicates poor growth or poor production. Where numbers were used the production was significant. Well diameter 1cm.

Starch casein solid media

Metals Fe (III) &	Modified CAS (Halo size, cm) Metal Concentrations (μM)									$FeCl_3$ CAS (Halo size, cm) Metal Concentrations (μM)								
	500	200	100	50	10	5	1	0.5	0.1	500	200	100	50	10	5	1	0.5	0.1
Mn II Growth	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Co II Growth	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	+	+	+	+	-	-	-	-	-	+	+	+	+
Cd II Growth	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	+	+	+	+	+	-	-	-	-	+	+	+	+	+
Ni III Growth	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	-	-	-	~	~	+	+	+	+	-	-	-	~	~	+	+	+	+
Al III Growth	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	~	+	+	+	+	+	+	+	+	~	+	+	+	+	+	+	+	+
Li I Growth	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	~	+	+	+	+	+	+	+	+	~	+	+	+	+	+	+	+	+
Cu II Growth	-	-	-	-	2.0	2.0	2.0	2.3	2.3	-	-	-	-	-	-	-	-	-
	-	-	-	-	+	+	+	+	+	-	-	-	-	+	+	+	+	+
Zn II Growth	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+
Mg II Growth	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Table 19: $FeCl_3$ and modified CAS assays of 23F growing in the present of iron and various other metals. – and + indicate the absence and presence (minor) of a halo respectively. ~ indicates poor growth or poor production. Where numbers were used the production was significant. Well diameter 1cm.

MS solid media

Metals	Modified CAS (Halo size, cm) Metal Concentrations (μM)									$FeCl_3$ CAS (Halo size, cm) Metal Concentrations (μM)								
	500	200	100	50	10	5	1	0.5	0.1	500	200	100	50	10	5	1	0.5	0.1
Mn II Growth	3	+	+	+	+	+	+	+	+	2.5	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Co II Growth	+	1.7	+	+	+	+	+	+	+	~	~	+	+	+	+	+	+	+
	~	~	~	~	+	+	+	+	+	~	~	~	~	+	+	+	+	+
Cd II Growth	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	-	-	~	+	+	+	+	+	+	-	-	~	+	+	+	+	+	+
Ni III Growth	~	~	~	~	+	+	+	+	+	~	~	~	~	+	+	+	+	+
	~	~	~	~	+	+	+	+	+	~	~	~	~	+	+	+	+	+
Al III Growth	-	-	-	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Li I Growth	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cu II Growth	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
	~	~	~	+	+	+	+	+	+	~	~	~	+	+	+	+	+	+
Zn II Growth	+	+	+	+	+	+	+	+	+	3.1	3	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mg II Growth	1.5 cm	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Table 20: $FeCl_3$ and modified CAS assays of 23F growing in the present of various metals. – and + indicate the absence and presence (minor) of a halo respectively. ~indicates poor growth or poor production. Where numbers were used the production was significant. Well diameter 1cm.

3. 16. 0. Some putative roles that can be ascribed to metal ions in Actinomycetes:

Clearly the actinomycete used here has an efficient mechanism (s) to allow ion acquisition. The specific metabolic roles played by the different metal ions are still unknown. However what is known is that metal ions influence morphological phenomena in actinomycetes (Beveridge *et al.*, 1997). In order to establish a link between the ions investigated in the study and morphology, a series of experiments were conducted using various concentrations of ions on confluently inoculated agar plates. Growth of cultures was observed over a three-day period. The strain's resistance to the metals has already partially been described in Tables 18 & 20. It was noticed that high concentrations of certain metals (Co II, Cd II, Ni III, Al III, Li I, Zn II) were toxic to the cells (Table 21). More specifically aerial mycelium formation was inhibited by supplementation of Co II, Ni III and Cu II (Figure 49).

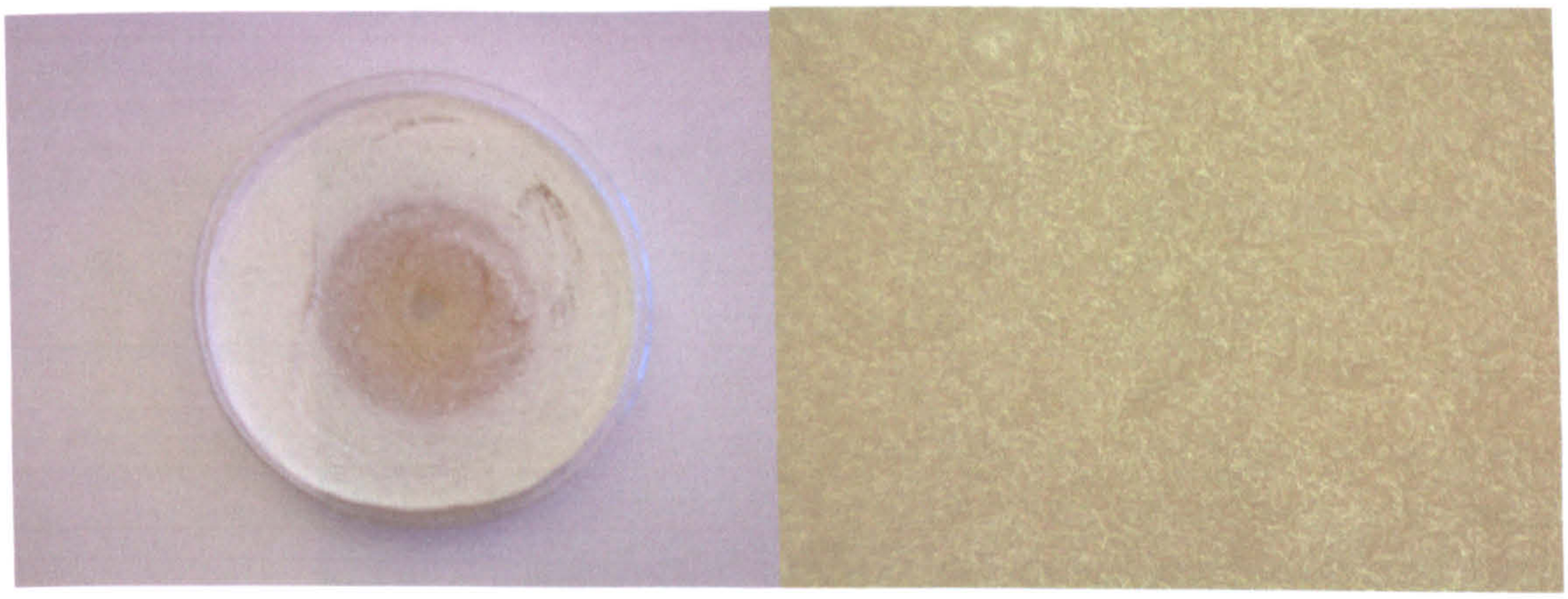


Figure 49: The affect of Co II on aerial mycelia formation (MS solid media). At 10-5 μM 23F grows around the well (left) but does not form aerial mycelia (right, light microscopy image).

On the other hand certain metals, such as Mg II, Fe III and Mn II, promoted growth. In the case of magnesium the highest concentration resulted in the greatest growth (Figures 50 & 51). That was not surprising, as numerous studies have identified this metal as an activator of enzymatic mechanisms. In *E. coli* the presence of potassium and magnesium alone is able to support fair growth with a small amount of iron growth promoting but not essential (Abelson & Aldous, 1950, Waring & Werkman, 1949 & Young *et al*, 1944).

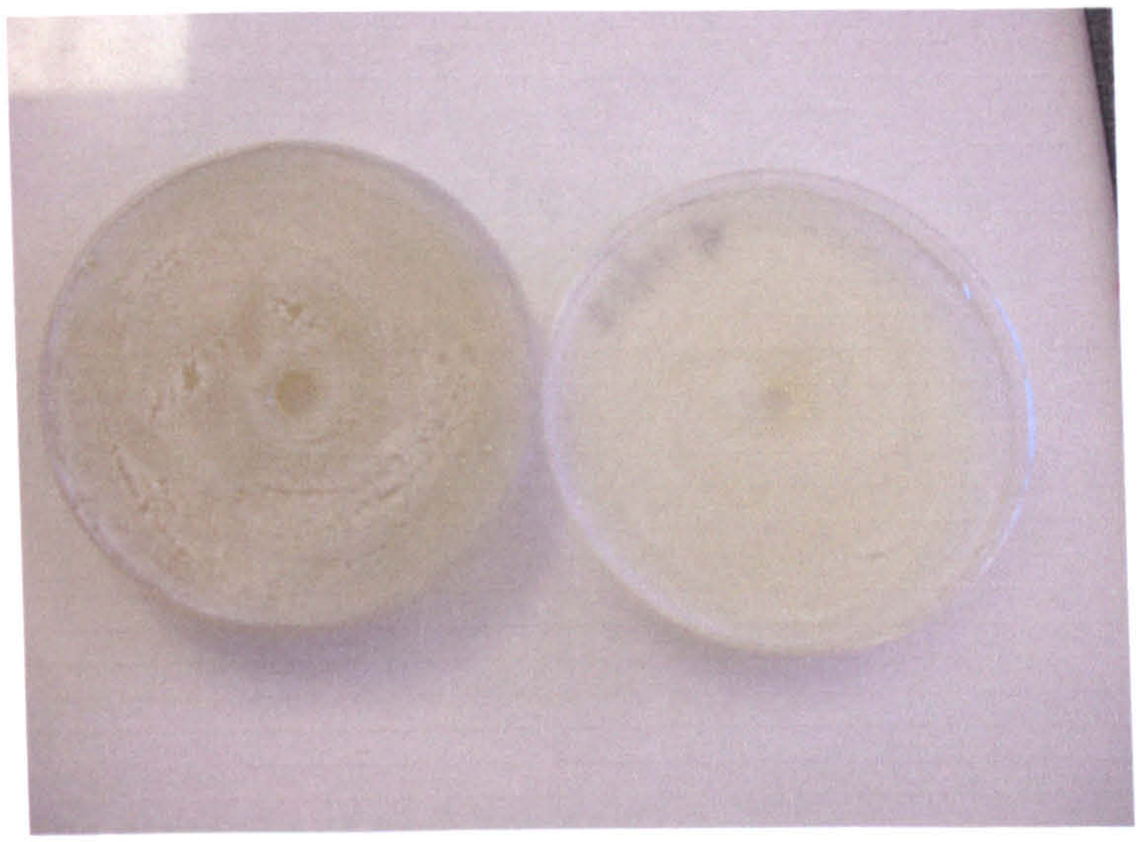


Figure 50: Comparison of the stain's growth in the presence of 500 μM (left) and 0.5 μM (right) of Mg II in starch casein media.

Figure 51: 23F culture growing the presence of 1 mM Mg II (MS).

Moreover the presence of 10 - 0.5 μM of Fe III facilitated growth demonstrating a significant improvement at cultures amended with a concentration of 0.5 μM of iron. However under high levels of iron (500 μM) light microscopy revealed the presence of substrate mycelia only (Figure 52).

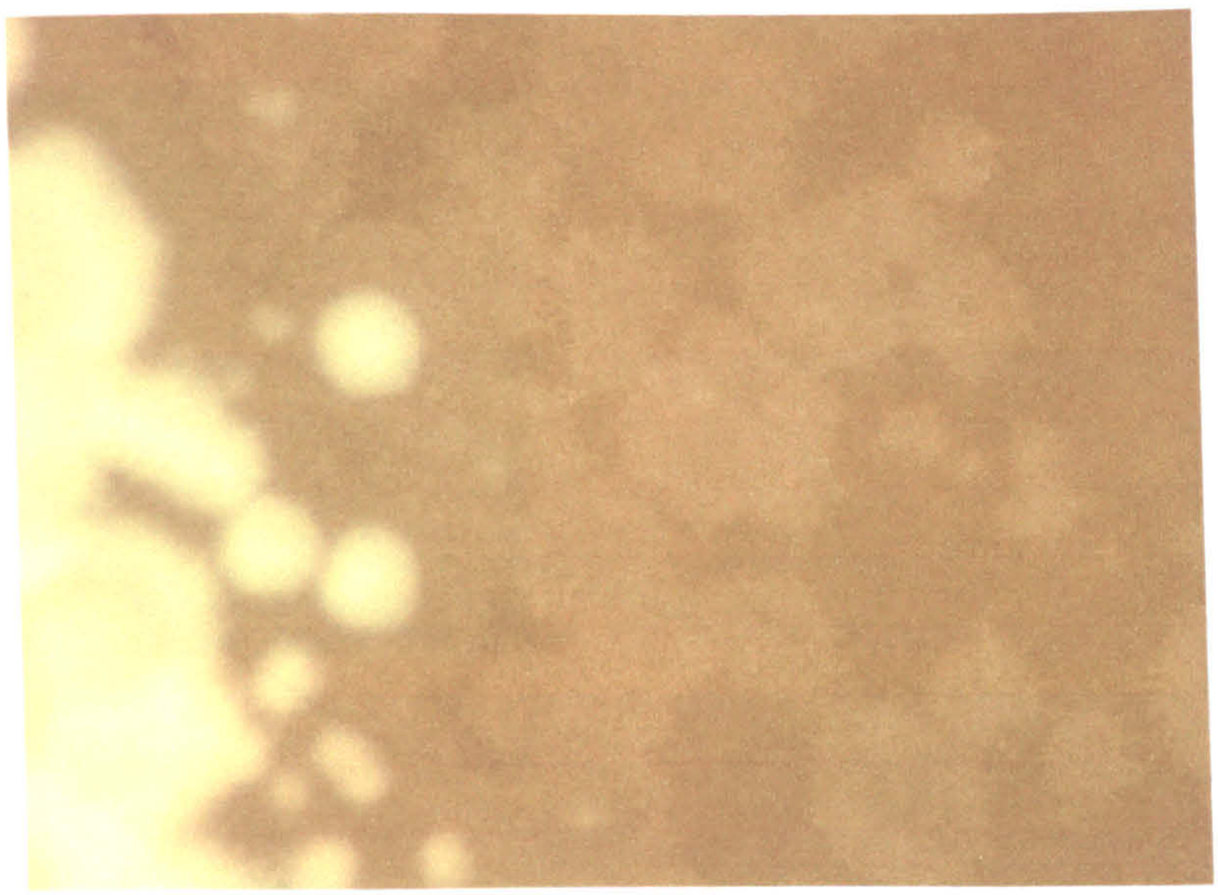


Figure 52: In the presence of high levels (500 μM) of iron in rich media (MS) only substrate mycelia were observed around the well.

It was discovered that although copper is very toxic, it has a physiological effect on the cells. The presence of small amounts (less than 0.5 μM) of Cu II in minimal media promoted the formation of aerial mycelia. Cells that were in direct contact with 0.5 μM of Cu II only developed substrate mycelia; however in a short distance from the well aerial mycelia were observed. This indicates that small quantities of copper may improve growth yields, suggesting a complex relationship between copper II concentration and differentiation (Figure 53).

In rich media (MS) at high levels of copper (1 mM- 10 μM) growth is inhibited. However as copper concentration declines away from the well substrate mycelia appear and grow in cord-like structures (Figure 54).

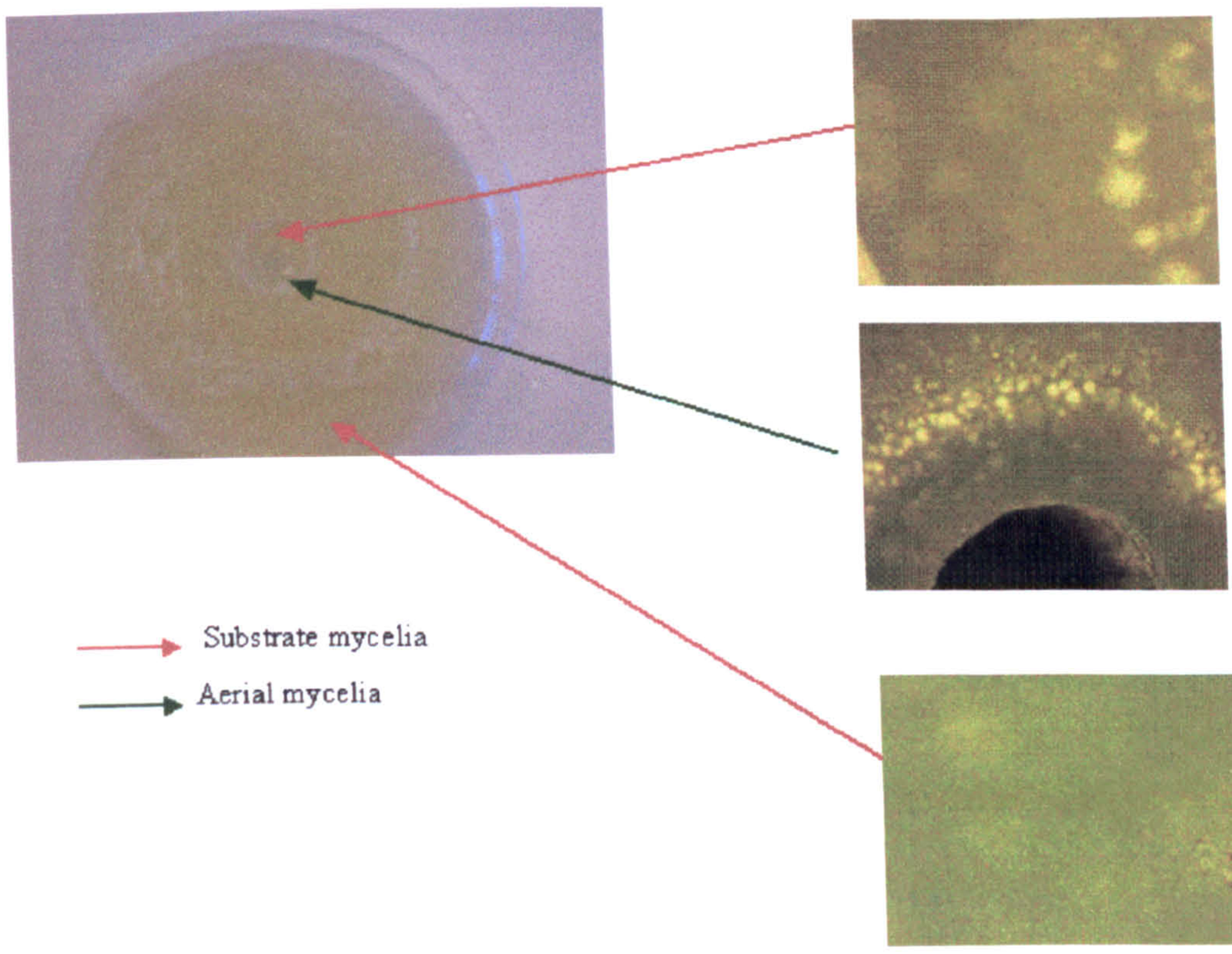


Figure 53: Description of the physiological effect of 0.5 μM of Cu II on 23F. As you move away from the well there is a zone of cells producing aerial mycelia. Further out from the centre aerial mycelia disappear and only a zone of substrate mycelia is evident. Moving further out of the centre a zone of aerial mycelia is again seen.

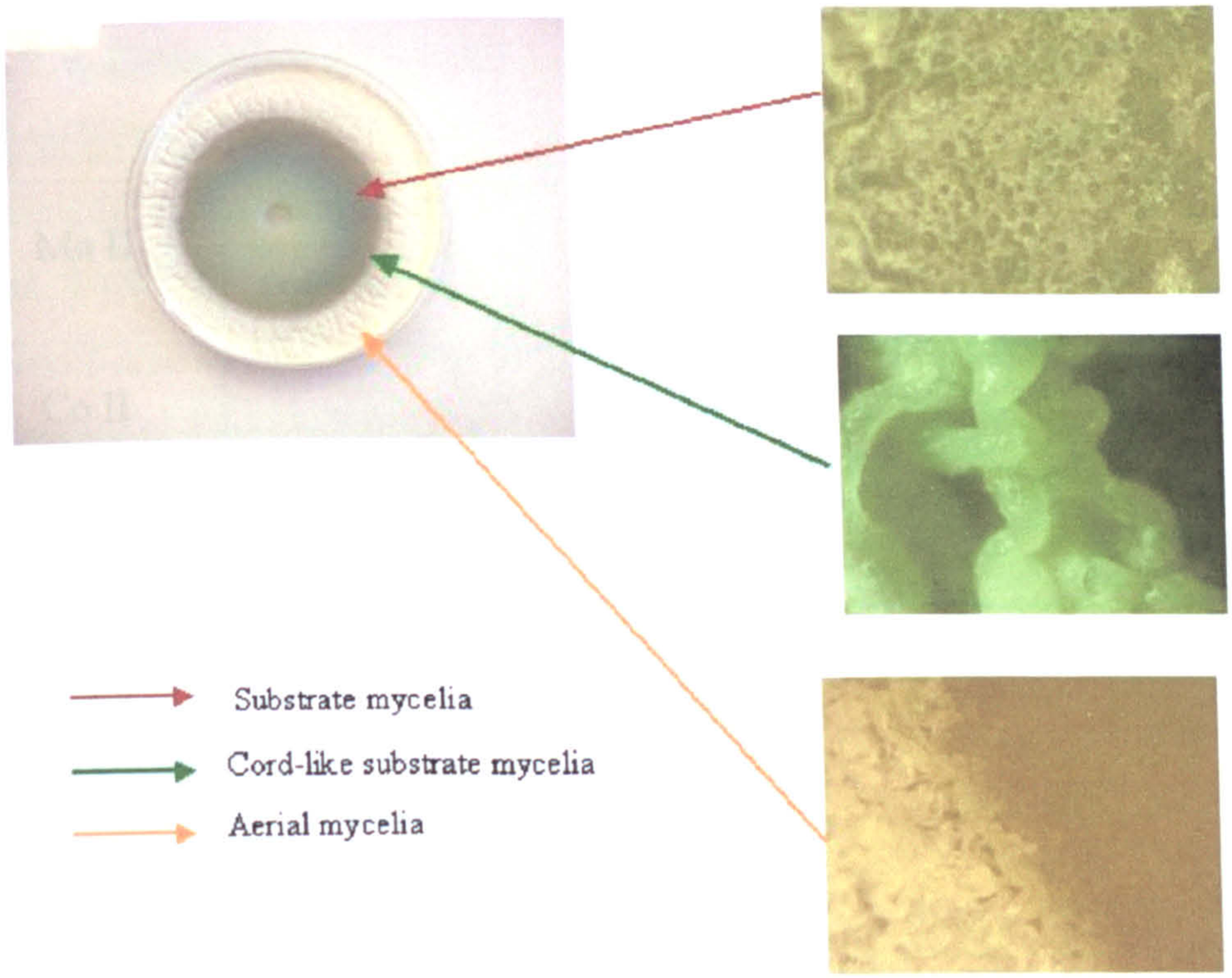


Figure 54: Description of the effect of 1 mM - 10 μM Cu II on the cells in rich media.

Mn II	Single colonies	~	~	~	+	+	+	+	+	+	+	+	+	+	↑	↑	↑	↑
Co II	-	-	-	-	-	-	Bold	Bold	Bold	-	-	-	-	Bold	Bold	Aerial mycelia	Aerial mycelia	Some aerial mycelia
Cd II	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ni III	-	-	Bold	Bold	~	~	~	~	~	-	-	Bold	Bold	~	~	~	~	+
Al III	-	-	-	-	+	+	+	+	+	-	-	-	-	↑ Sporulation	+	+	+	+
Li I	-	-	-	↑	+	+	+	+	+	-	-	-	↑	+	+	+	+	+
Cu II	-	-	-	-	-	-	-	Bold		-	-	-	-	-	-	Bold	Bold	Bold
Zn II	-	-	-	-	~	+	+	+	+	-	-	~	~	~	+	+	+	+
Mg II	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑
$FeCl_3$	-	-	-	-	↑ Sporulation	↑ Sporulation	↑	↑	↑	-	-	-	-	↑ Sporulation	↑	↑	↑	↑ <u>Best</u>

Table 21: Description of the effect of different of different ion concentrations on the isolate's physiology.

+ and - indicate growth and the lack of it respectively

3. 17. 0. Gene mining and comparative genomics:

Part of this work involved the identification of the desferrioxamine E gene in the strain 23F's genome with the intention to perform PCR directed gene disruption (Gust *et al.*, 2002). This would then allow us to relate the gene with physiological functions. It would also contribute towards understanding the molecular control of the siderophore genes in actinomycetes.

When this work was conducted four complete genome sequences of actinomycetes were available in the public domain, *Streptomyces coelicolor*, *Streptomyces avermitilis*, *Mycobcterium tuberculosis* and *Nocardia farcinica*.

According to data published by Yamanaka *et al.*, (2005) two DNA fragments, a and b, that correspond to internal regions of *desA* and *desD* were amplified from the chromosomal DNA of *S. coelicolor* (Figure 55). The primers employed for the PCR amplification of these regions were: 5'-CGACAAGCTTGAGGACGTCTA-3' (3035832-3085852 of SCO2782) and 5'-AAGGAGATCTCCCAGGTACT-3' (3037049-3037068 of SCO2782) for a and 5'-CTTCGAGATCTACGAGTACCT-3' (3039416-3039436 of SCO2785) and 5'-CAGGGAATTCTTCAGGGTAC-3' (3040650-3040670 of SCO2785) for b.

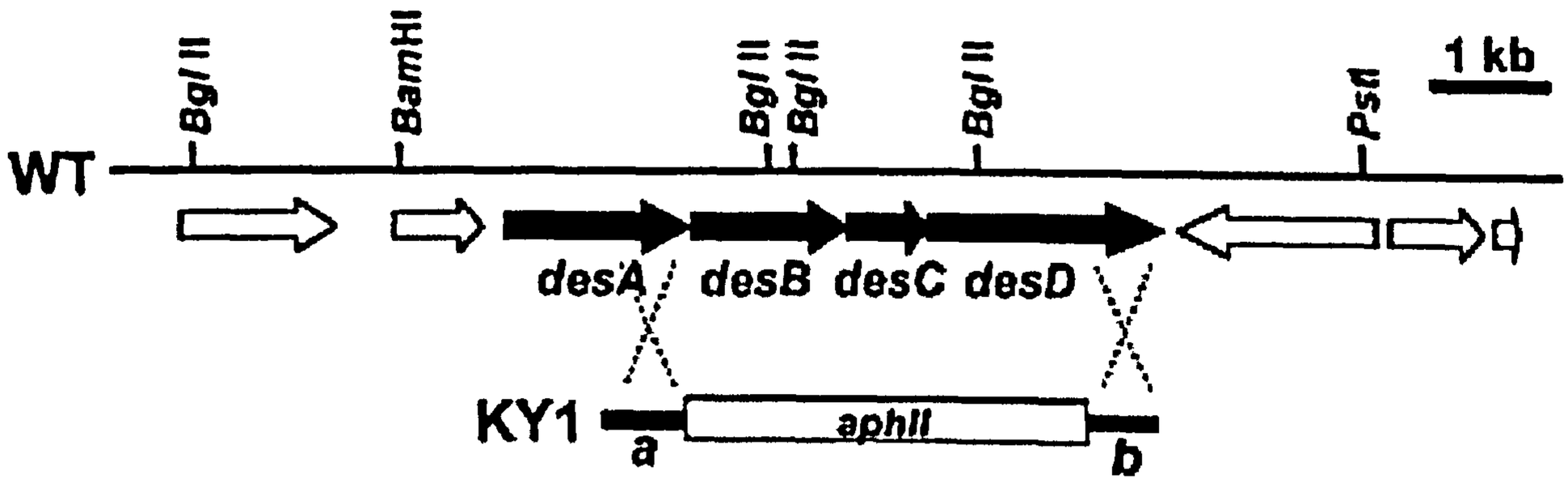


Figure 55: Schematic representation of desferrioxamine biosynthesis gene cluster of *S. coelicolor* A3(2). Redrawn from Yamanaka *et al.*, 2005.

DNA from 23F and *S. coelicolor* M145 was extracted (Kirby Mix procedure) and visualised on a 1% agarose gel following a 48 hour incubation in an orbital shaker at 30^0 C, 250 rpm. PCR amplification was conducted using the primers of Yamanaka *et al.*, including positive controls (StrepB-StrepE and StrepB-StrepF) and a negative one (no DNA). PCR amplification was performed with a Perkin-Elmer Cetus GeneAmp Thermal Cycler, 9600, using 35 cycles of 95^0 C for 1 minute, 52^0 C for 1 minute and 72^0 C for 2 minutes. Reaction mixtures contained 45 µl of 1.1 x PCR MasterMix (1.5 mM $MgCl_2$) [ABgene], 2 µl of DMSO, 1 µl of genomic DNA, 1 µl of sterile dH_2O and 0.5 µl of each primer (forward and reverse). PCR products for the positive controls, visualised on an ethidium-bromide-stained 2 % agarose gel, were of the predicted size, confirming a successful amplification; however the *desA* and *desD* products appeared to be larger than expected.

Careful examination of the work by Yamanaka *et al.*, 2005 revealed various factual inaccuracies leading to the conclusion that the design of the primers was not fit for purpose.

The next stage involved the mining of *S. coelicolor*'s homologous *desA* and *desD* gene sequences. The retrieval of the sequences was conducted by the NCBI BLAST database (available online- http://www.ncbi.nih.gov/) and involved all four fully sequenced actinomycetes. Multiple alignments using BioEdit sequence alignment Editor 7.0.4.1. (Copyright 1997-2005, Hall, 1999) revealed that the siderophore biosynthetic genes *desA* and *desD* were not highly conserved amongst the actinomycetes.

S. coelicolor and *S. avermitilis* were found to be most closely related presenting an 84 % and 90 % similarities for *desD* and *desA* sequences respectively (7. 2. 0. / Appendix III / sequence alignments).

Following careful examination of the pairwise sequence alignments (NCBI BLAST database, available online- http://www.ncbi.nih.gov/) of *S. coelicolor* and *S. avermitilis*, specific primers targeting *desA* and *desD* sequences were designed using the most conserved regions of the genes (Table 22). The primers were nested to ensure good quality of the gene sequence and the largest product possible.

Gene targeted		Primers
desA	Forward 1	5'-TCCGCGACGCCGTCTACTTCC-3'
	Reverse 1	5'-GGCGAGGAGCAGCGCCTGGA-3'
	Forward 2	5'-TCGACGCGGCCTACGGCTGC-3'
	Reverse 2	5'-TGAGGTACTCCGCGTGGTAGG-3'
	Reverse 3	5'-CACGCGCAGCGTCATCCACA-3'
desD	Forward 1	5'-GAGGAGATCTCCTCCACCCT-3'
	Reverse 1	5'-GGCTCGCGGTCTCGGGGGCG-3'
	Forward 2	5'-ACGACGAGTACCTGGCCCAG-3'
	Reverse 2	5'-TCAGCGCGCCCGCGAAGGAG-3'
	Reverse 3	5'-GTCGAAGACGTCCGTGAAGA-3'

Table 22: Description of the nested primers designed to target *desA* and *desD* gene sequences of the desferrioxamine biosynthetic gene cluster.

For *desA* the size of the PCR products expected were as follows:

Forward 1-Reverse 1 ⟶ 239 nt

Forward 1-Reverse 2 ⟶ 731 nt

Forward 1-Reverse 3 ⟶ 836 nt

Forward 2-Reverse 2 ⟶ 167 nt

Forward 2-Reverse 3 ⟶ 272 nt

For *desD* the size of the PCR products expected were as follows:

Forward 1-Reverse 1 ⟶ 182 nt

Forward 1-Reverse 2 ⟶ 855 nt

Forward 1-Reverse 3 ⟶ 1136 nt

Forward 2-Reverse 2 ⟶ 372 nt

Forward 2-Reverse 3 ⟶ 662 nt

PCR amplification of DNA extracted from both *S. coelicolor* M145 and 23 F was repeated (same conditions) and in most cases one single product of the predicted size was visualised. *S. coelicolor* M145 was introduced as a control and in all reactions amplifications were successful. PCR products for 23F's *desD* gene Forward 2-Reverse 2 and Forward 2- Reverse 3 consisted of 2 bands including one of the predicted size in both cases (372 nt and 662 nt respectively). Although these PCR reactions were not included in any further studies they could prove valuable in future experiments (gene disruptions). The remaining PCR products were sent to Lark$_{TM}$ Technologies, Inc (United Kingdom) for sequencing.

Sequences of *S. coelicolor*'s *desA* and *desD* revealed 100 % homology to the ones published in the organism's sequenced genome database (The Wellcome Trust, Sanger Institute, available online: http://www.sanger.ac.uk/) confirming that the correct genes were targeted. The sequenced *desA* gene (Figure 56) of 23F's strain revealed 84 % homology to *S. avermitilis*'s siderophore biosynthetic gene cluster, lysine decarboxylase, (identities 741/878, 5 % gaps) and 83 % homology to *S. coelicolor*'s putative pyridoxal-dependent decarboxylase, *desA*, (identities 732/878, 4 % gaps). The sequence retrieved for 23F's *desD* (Figure 57) showed 82 % similarity to *S. avermitilis*'s siderophore biosynthetic gene cluster (identities 165/199, 7 % gaps).

These results demonstrate successful gene mining of the desferrioxamine's (E) gene cluster, which will facilitate the identification of the specific role of each gene in the chelating agent's biosynthetic pathway. This will further enable the monitoring of the gene expression at the level of messenger RNA. These primer pairs will provide important diagnostic tools for any future studies on transcriptional regulation of these genes; although this was not intended to be part of this particular project.

This approach could also examine the effect of disrupting the transcriptional repressor DmdR. Successful disruption of this gene is likely to result in a phenotype that expresses the hydroxamate in the presence of excess iron, the co-repressor of the siderophore biosynthetic pathway. Biotechnologically this will be important as such a strain will enable constitutive production of the commercially valuable biomolecule.

DesA/ 23F (878 nt)

CCGCGACGCCGTCTACTTCCACCACCCGCGCTACCTCGCCCACCTCAACTG
CCCGGTCGTCATCCCGGCCGTCCTCGGCGAGGCGGTCCTCTCGGCGGTCA
ACTCCTCCCTGGACACCTGGGACCAGTCGGCGGGCGGCACCCTCATCGAA
CGGCGCCTCATCGACTGGACGGCCGGCCGCATCGGCCTCGGCGAGAGCGC
CGACGGCGTCTTCACCAGCGGCGGCTCGCAGTCCAACCTCCAGGCCATGC
TGCTCGCCCGCGAGGAGGCCAAGGCAGCGCACCCCACCCAGCTGCGCGTC
TTCACCTCCGACGTCAGCCACTTCAGCGTCCGCAAGTCGGCGACGCTGCTG
GGTCTCGGCCCGGACGCCGTGGTGGTCGTCCCCACCGACCGCGAGCGCCG
CATGCGGATCGAGGTGCTCGCCGCCGAGCTGCGCCGCTGCGAGGCCGAGG
GCCTGGTGCCGATGGCCGTCGTCGGCACCGCCGGCACCACCGACTTCGGC
TCGATCGACCCGCTCCCGGAGATCGCCGAACTCTGCGCCCGCCACAACAC
CTGGATGCACGTCGACGCCGCGTACGGCTGCGGACTGCTCGTCTCCCCCA
CCAGGCGCGGACGGCTCACCGGCGTCGAGCACGCCGACTCGGTCACCGTC
GACTACCACAAGTCGTTCTTCCAGCCGGTGAGTTCCTCCGCCCTGCTGGTC
CGCGACGGCACCACCCTGCGGCACGCCACGTACCACGCGGAGTACCTCAA
CCCGCGCCGGGCCGCCGAGGAGCGCATCCCCAACCAGGTCGACAAGTCGC
TTCAGACCACCCGCCGCTTCGACGCCCTGAAACTGTGGATGACGCTGCGC
GT

Figure 56: The nucleotide sequence of *DesA* from isolate 23F.

DesD/23F (199 nt)

GGAGATCTCCTCCACCCTCTCCGGCGCCGCCTGGAAGCTGGCCCAGGCCC
GCCCCAGCGCCGCCGAGCTGGCCCGCGCCGGCTTCCAGGCGATCGAGACG
GGGATGACCGAGGGCCACCCCTGCTTCGTCGCCAACAACGGGCGGATCGG
CTTCGACGTCCGCGAGTACCACCAGTACGCCCCCGAGACCGC

Figure 57: nucleotide sequence of *DesD* from isolate 23F.

3. 18. 0. Bioremediation / immobilization:

In order to explore the potential biotechnological applications of isolate 23F it was necessary to achieve successful immobilisation of the organism in a model bioreactor, under suitable conditions that would not affect siderophore production. This would evaluate whether strain 23F could be applied in an environment suitable for industrial bioremediation. The rationale for this approach was to develop a fluidised bed model reactor that could be used to remove soluble ions from contaminated water sources, such as local polluted canal and river waters. A prerequisite for such a system is that the biomass should be immobilised within the bioreactor system.

Therefore a series of "inert" porous supports were investigated for their ability to sustain immobilised growth of 23F and siderophore production with a view to using this system in bioremediation.

3. 18. 1. Pumice stones:

An ideal matrix for use in a fluidised bed reactor is one that is relatively light; hence ensuring minimum energy input to maintain the bed in a fluidised manner. One such support is pumice stone, a natural product of volcanic origin. Pumice stone is light with the advantage of having a large rough surface area for cellular attachment. The initial tests were carried out in shaking cultures containing, YEME, MS and starch-casein media separately, where pumice stones demonstrated degradation due to friction. In order to rectify this problem a new set of liquid cultures were incubated statically at 30^0 C for nine days. Observational analysis of immobilisation using the dissecting microscope appeared to be inconclusive. The reason behind this was that all samples of pumice stones, including the control, appeared to be similar. It was difficult to determine if the isolate had indeed immobilised onto the stones or if media components had accumulated into the porous surface.

Further investigation included Gram staining. All the pumice stones, including the control, stained purple. Therefore it was not possible to determine if the dye had actually stained the bacteria or was absorbed by the porous stones.

Since the methods employed did not show any indication of immobilisation it was decided to use scanning electron microscopy (SEM). Stones from YEME, MS and starch-casein cultures (4 days old) were analysed under the SEM, where it was evident that 23F was immobilised onto the pumice in all three cases (Figure 58).

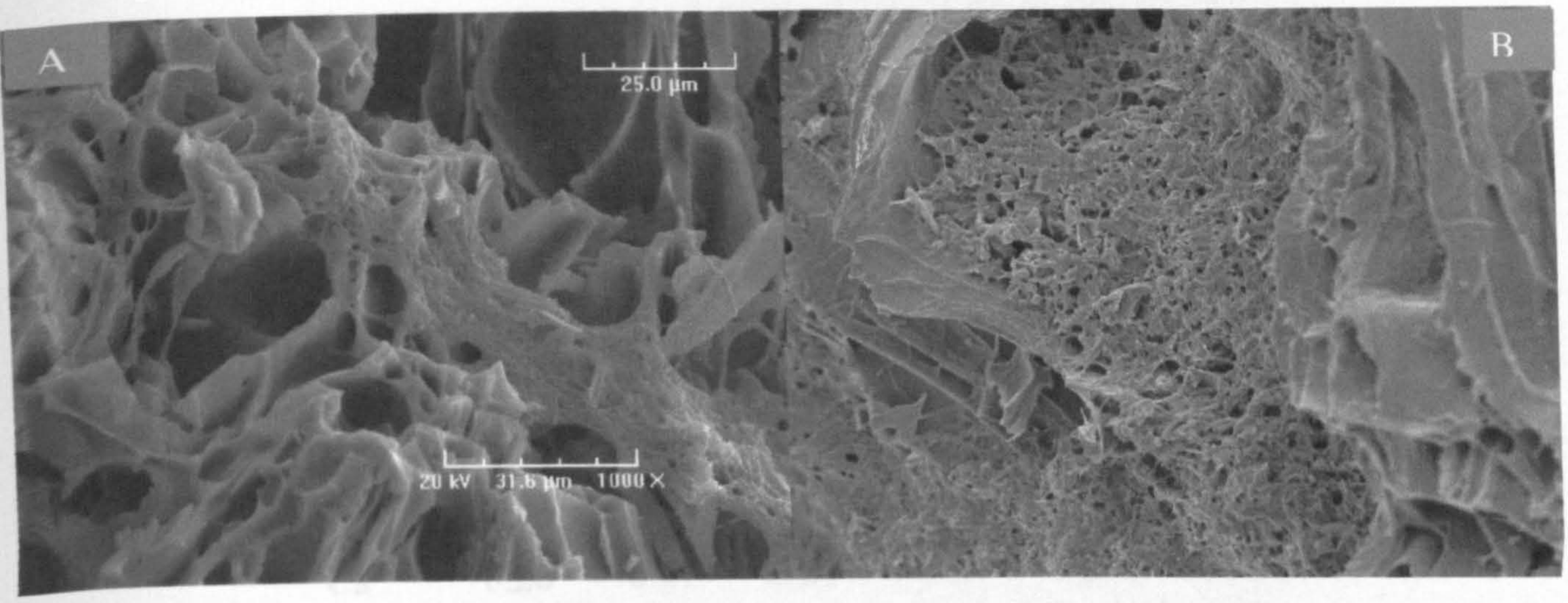


Figure 58: Scanning electron microscopy demonstrating immobilised 23F into pumice stones in MS (A) [x 1000 magnification] and YEME (B) [x 500 magnification] cultures.

Once immobilisation of 23F was successful, the ability of the organism to synthesise and release siderophores was investigated. In all media tested no siderophore production could be measured. In order to further investigate the suppression of the iron chelating agents, X-ray analysis was performed on various stones.

Their elemental composition and spectrum analysis are included in Appendix II. A brief summary is included in Table 23. All of the pumice stones (PS) analysed, PS1 to PS6, contained numerous elements such as oxygen (O), sodium (Na), aluminium (Al) and iron (Fe); however their composition varied considerably.

Natural iron was an important discovery in the pumice stones as siderophores are repressed in the presence of certain concentrations of this specific ion; all of the pumice samples analysed contained iron within the range of 0.29 to 1.34 Wt %.

In an attempt to remove the iron from the pumice stones, they were left overnight in a 10 mM Dip solution (iron chelating agent). Following Dip removal by rinsing, X-ray analysis was repeated and revealed the presence of iron once again. Although pumice stones demonstrated a very efficient immobilising support, they clearly were not inert and we no longer pursued for this purpose. It is worth noting that very efficient immobilisation was achieved with this technique and although this was not fit for siderophore biosynthesis, it may well be an ideal matrix for other purposes, such as secondary metabolite production. Therefore different surfaces had to be explored.

Elements (Wt %)	PS1	PS2	PS3	PS4	PS5	PS6
B	11.6	9.59	18.48	-	-	6.11
C	12.62	16.96	-	7.53	13.10	14.14
O	67.18	69.59	60.28	17.67	56.55	65.35
Na	0.61	0.36	1.33	1.16	1.86	0.85
Mg	0.05	0.08	0.07	-	0.10	0.02
Al	1.33	0.63	3.05	3.24	3.98	1.44
K	0.51	0.05	1.42	-	1.41	0.50
Ca	0.20	0.27	0.48	0.54	0.82	0.36
Fe	0.48	0.73	1.20	1.34	0.92	0.29

Table 23: A very brief elemental composition, given as weight (Wt %), of various pumice stones (for more information please refer to Appendix III).
(-) Signifies element not present.

3. 18. 2. Coal:

The next surface trialed for the immobilisation of 23F was coal. In order to establish the elemental composition of the specific surface, an X-ray analysis was performed on a coal (CO) sample (Appendix IV). The presence of iron was detected to be of a value of 0.16 (Wt %), which was lower than pumice stones. Additionally the sample had a high concentration of carbon, 26.79 (Wt %), which was expected, as it is a natural fossil fuel. The coal was tested directly in starch-casein liquid cultures inoculated with 23F and no siderophore production was detected. The control, which contained no coal, resulted in an orange halo of 2 cm diameter confirming the presence of hydroxamate siderophore. The well's diameter was 1 cm. Although coal represented a natural surface and a powerful immobilising agent, it was not investigated any further. Like pumice stones coal could be applied in bioremediation but its efficiency would prove limited because of the presence of iron in the support matrix.

3. 18. 3. Sponge:

The next trial involved two kinds of synthetic, porous sponge (SP): the green (SP1) and the aqua coloured kitchen sponge (SP2). Both types were analysed with respect to their elemental composition (Appendix IV), which revealed that neither of them contained any iron.

Both SP1 and SP2 were trialed with 23F in starch-casein liquid medium and tested on CAS agar. The organism, 23F, was immobilised in both sponges. Neither of the samples affected or inhibited hydroxamate production, as a 2 cm diameter orange halo was observed in each case, including the control. The wells were each 1 cm in diameter.

When 23F was grown in MS liquid medium immobilisation in the sponges was observed to be substantially increased. Although the organism was immobilised in both sponges, SP2 appeared to provide a better surface (Figure 59). SEM analysis of the two sponges also confirmed excellent immobilisation of the isolate and SP2 was consistently found to offer a better surface than SP1 (Figure 60).

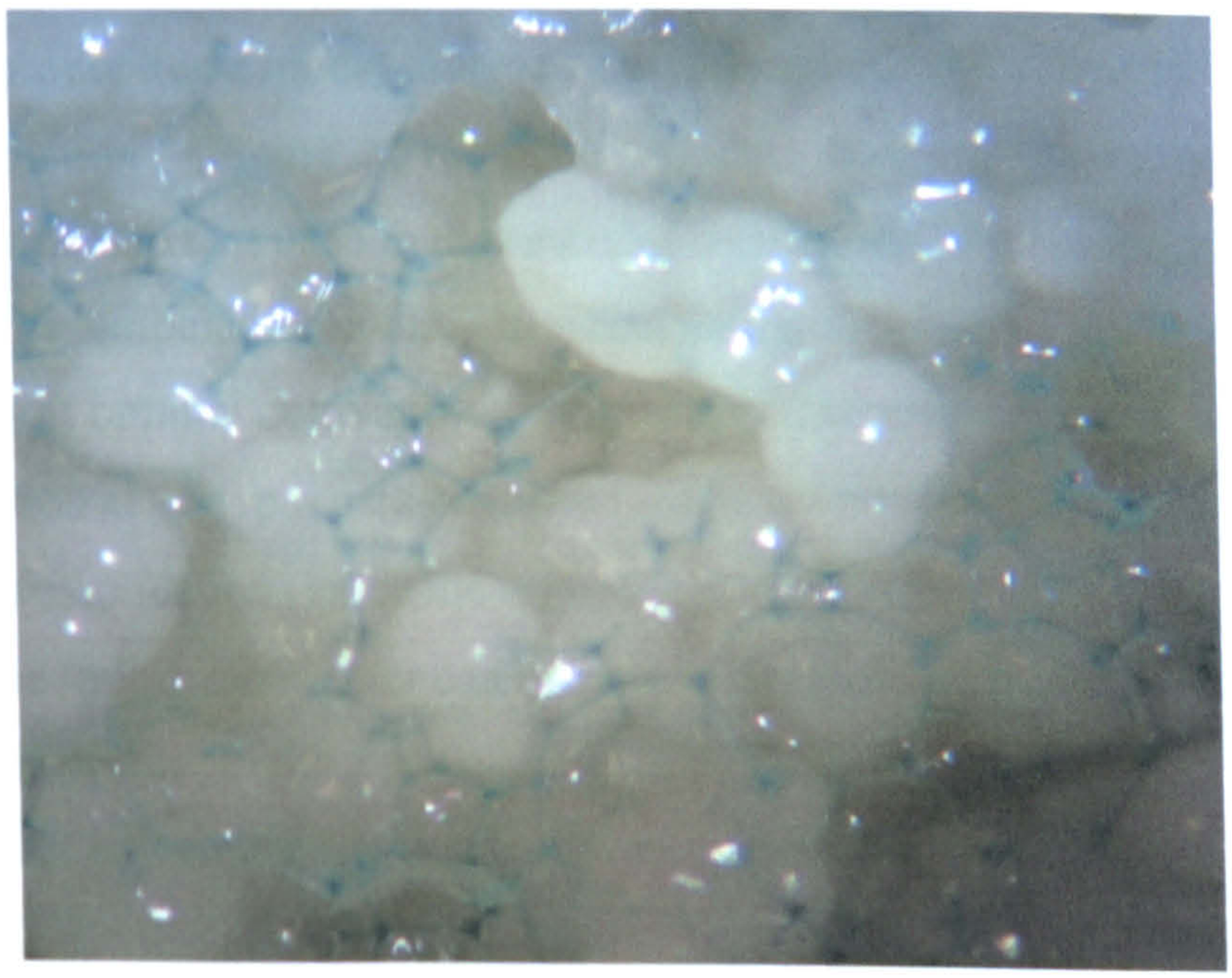


Fig. 59: Photograph demonstrating successful immobilisation of 23F in the aqua coloured kitchen sponge (SP2). The picture was taken with the dissecting microscope and the organism was cultivated in MS liquid media at 30 °C for two days.

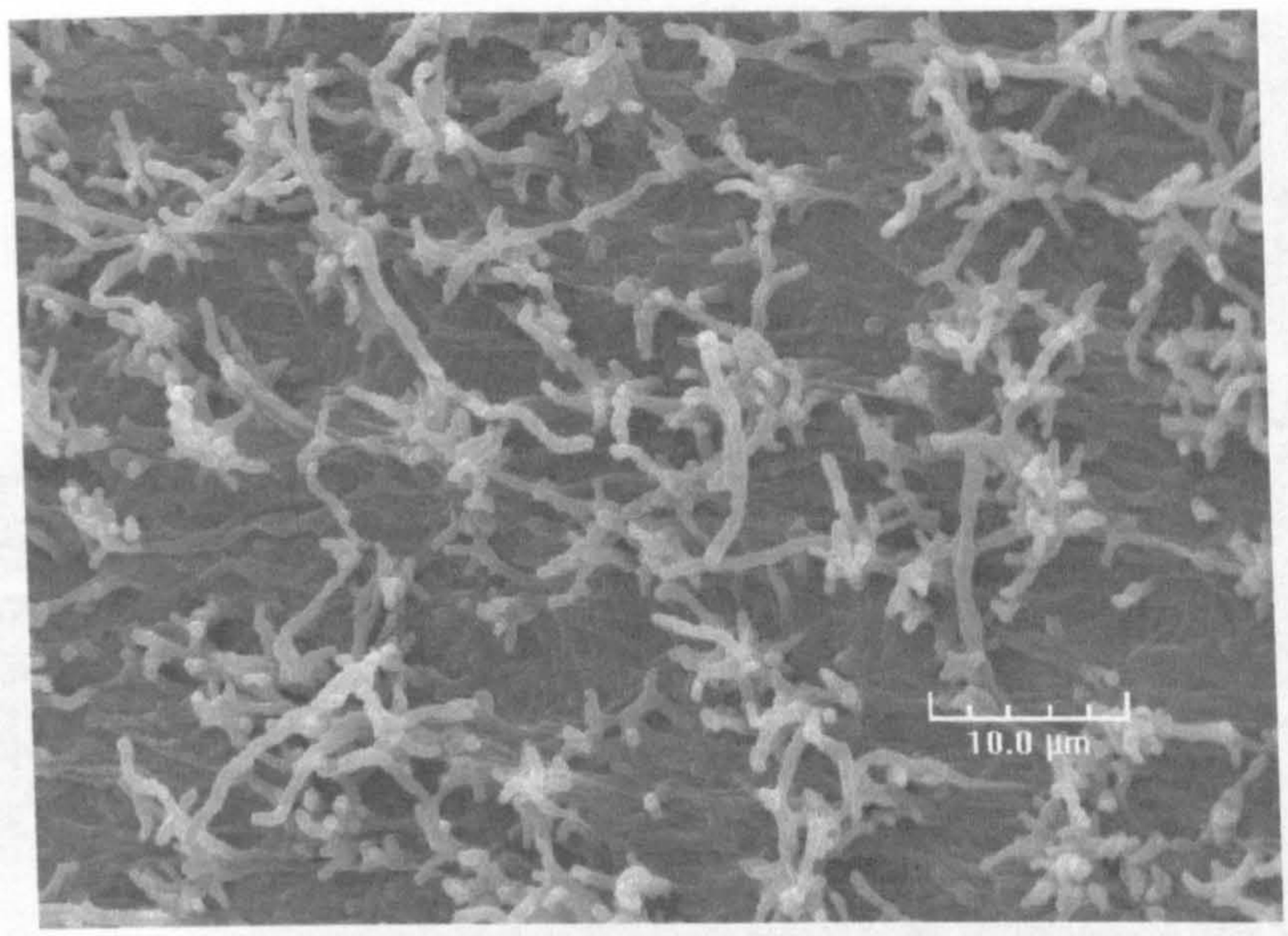


Fig. 60: Scanning electron microscopy image (x 2000 magnification) of 23F immobilised within SP2. The organism was grown in liquid MS media for two days at 30° C.

3. 18. 4. Model bioreactor:

Following successful immobilisation of 23F on SP2 sponge without affecting siderophore production a model bioreactor was designed with a view to its application in bioremediation (Figure 61). The model had to represent a similar microenvironment to that used in commercial fermenters. Therefore certain important factors, such as temperature control, aeration and agitation, had to be incorporated in order to support optimal cultivation of the isolate. The temperature was kept at 30^0 C and the agitation at 250 rpm using a heated stirring plate. The process was run pH free to minimize complications since the organism grows from pH 5 - 9. Depending on the bioprocess the 'micro' fermenter can be potentially adjusted to include pH control via an appropriate probe.

The bioprocess involved a fed batch culture (starch-casein liquid media), previously described in Materials and Methods section. The working model efficiently supported the growth and immobilisation of 23F. The CAS assay was applied daily in order to monitor siderophore production, which was only detected after the addition of the feed on the third day. The chelating agent was identified as a hydroxamate, which was later confirmed to be desferrioxamine E by TLC.

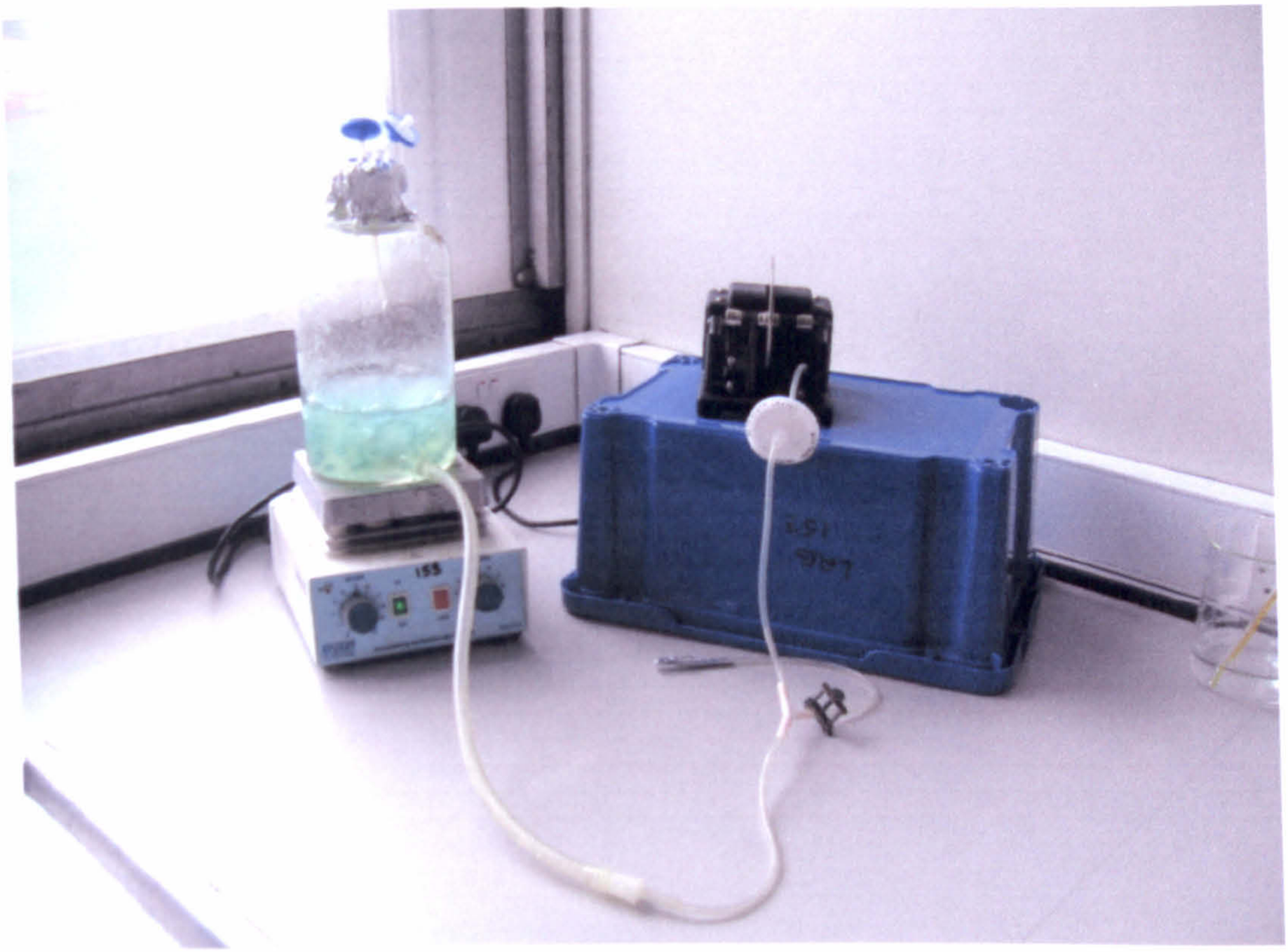


Figure 61: The bioreactor model, which includes the heated, magnetic stirrer, as seen in the diagram and inoculated (23F) starch casein medium. Aqua coloured kitchen sponge is also present.

The results above demonstrate that 23F is an ideal candidate for immobilisation due to its ability to grow fast and form tight pellets. Moreover aqua coloured kitchen sponge offers a relevant porous support for excellent immobilisation. However, another approach would be to use natural sponge as it has previously demonstrated its effectiveness in immobilising various bacterial species (Vignoli *et al.*, 2006). Additionally natural sponge is degradable and therefore would not be detrimental to the environment.

Although this system is not applicable for 'direct' iron removal, as the presence of this ion would repress siderophore production, it is ideal for copper decontamination due to the fact that its acquisition by the specific chelating agents is independent of the Fe sequestering mechanism. Copper toxicity is a particular problem at copper wire and coin manufacturing sites. Furthermore in the absence of iron this model will be excellent for bioremediation of Mn (II), Co (II), Cd (II), Ni (III), Al (III), Li (I), Zn (II) and Mg (II).

Moreover this system could be further developed to include iron bioremediation. This would involve the cultivation of the isolate in a large scale scale bioreactor allowing

the hydroxamate to be released, which will then be applied/ sprayed onto the iron contaminated area. Alternatively it could be explored for the production of catechol based siderophores as it appears that up to certain concentrations of iron the release of this siderophore is not affected.

Chapter 4

Conclusions:

In an attempt to identify novel siderophores with a view to commercial exploitation and to develop a better understanding of the physiological roles of these compounds we have isolated 154 streptomycetes from soil samples collected in Thailand (Tak province) and the UK (Freshfield). Twenty- two Thai and five UK strains were confirmed CAS-assay positive exhibiting prolific siderophore production.

In order to determine the classes of siderophores expressed the colorimetric assays of Arnow (for catechols) and Atkins (for hydroxamates) were applied. Originally in two cases (samples 5 and 6 from Freshfield) both hydroxamate and catechol type moieties were produced, supporting the contingency theory. The latter suggests that members of streptomycetes produce multiple siderophores, which are independently regulated and act contingently in order to compete more efficiently in their environment.

In order to protect the intellectual property of the potentially valuable siderophore producers, analysis of five nucleotide sequences of 16S rRNA was performed and the taxonomic relationship of these isolates (23F, 29C, 31B, 31C and 33D) was reported using ClustalX.

To establish the novel nature of the strains that have been screened a variety of morphological, cultural, physiological and biochemical characteristics were determined to produce a detailed phenotypic and metabolic profile. This involved light microscopy and scanning electron microscopy (SEM) along with growth optimisation, Biologs, Mastrings and susceptibility discs.

The ability of the isolates to over produce siderophores was also verified by growth in the presence of high concentration of 2, 2'- Dipyridyl (DIP). The latter is a powerful iron-chelating agent that scavenges ferric complexes from the environment sending a false signal of iron starvation. It is believed to apply selective pressure for organisms adapted to iron deficiency, i.e. by those that possess siderophore biosynthetic mechanisms.

Stains 31B, 31C and 29C were proven to have fastidious growth requirements; although a catechol overproducer 29C still attracts interest due to its capacity to scavenge iron on the CAS assay. Isolate 33D appeared to be a fast grower and easy to cultivate; however its physiological status, which manifested in the formation of big spherical pellets (approximately 2.5 mm), would have obstructed siderophore production in submerged cultures using bioreactors. When the organism was grown in liquid media the pellets' centre suffered nutrient and oxygen limitation leading to subsequent death.

Isolate 23F was categorised as a multiple siderophore producer, synthesizing both a hydroxamate (evidenced by HPLC, TLC and CAS assay analysis) and a catechol (CAS assay), depending on the availability of nutrients and ions in its surrounding. In rich MS media the strain synthesises a catecholic compound, which forms strong and extremely stable ferric complexes, yet to be further characterised. In starch casein basal media the isolate produces a hydroxamate, classified by HPLC as desferrioxamine E. The latter is a valuable compound having both clinical and environmental applications. A very recent study investigated the use of the biomolecule in drug delivery therapies by 'creating' conjugates with metabolic inhibitors, such as antibiotics.

Both of the siderophores produced by 23F are structurally distinct and they appear to be regulated by two independent biosynthetic pathways. This mechanism has possibly evolved in the strain to out-compete in its natural environment and eliminate the piracy of its chelating agents by microorganisms, which cannot synthesise them but can recognise, transport and utilise them as xenosiderophores.

Moreover production of the siderophores in the strain is regulated by different amounts of iron availability. It appears that supplementation with iron (100 μM $FeCl_3$) partially represses the biosynthetic pathway of the catechol as opposed to desferrioxamine's, where even in concentrations of $FeCl_3$ of 0.2 μM, the hydroxamate production is completely inhibited. Therefore we can suggest that relatively high levels of iron (100 μM $FeCl_3$) still facilitate the biosynthetic pathway of the catechol, whereas it completely represses hydroxamate siderophores. The opposite system has been observed with the cyanobacterium *Oscillatoria temuis* Ag. UTEX 428, which in the presence of 4.1×10^{-7} M $FeCl_3$ produces a hydroxamate-type siderophore, whereas a catechol-like one is produced at lower levels (Wilhelm & Trick, 1994). Hence we can propose that the iron regulation and transportation system is strain dependent and not siderophore specific.

In an attempt to improve the desferrioxamine E production a fed batch system was employed and there is significant evidence to believe that by feeding supplements (amino acids and Dip) to the fermentation we can increase the yield of the specific siderophore, directing the bioprocess toward the desired product. Besides lysine the remaining amino acids tested are not obvious direct precursors to the specific compound; however they do play a role in its biosynthesis. Therefore we propose that they are more readily transported by the organism than lysine and are then subjected

to transamination/ dehydration reactions leading to the generation of the lysine precursors.

Furthermore it appears that the combination of glutamic, aspartic acids and serine also have a positive effect on the strain's viability providing a perfect model for a robust system, where desferrioxamine E would be fully controlled and produced at a constant rate. This work illustrates how careful screening techniques followed by judicial medium design can be successfully employed to produce bioactive molecules.

A very important finding of this study was the production of desferrioxamine E in rich media (MS), where only the catecholic compound is known to be naturally expressed. In the presence of lysine catechol production appeared to be almost unaffected, however the organism synthesised the second siderophore in the presence of the biomolecule's precursor. This also supports the contingency theory by confirming the existence of two independently regulated (siderophore) biosynthetic pathways.

Furthermore it was noticed that the provision of certain supplements did influence the strain's physiology without affecting the siderophore production. The later implies that morphological differentiation (fragmentation, pelleting and sporulation) does not appear to be associated with the chelating agent's synthesis.

Although the organism produces the specific compounds (siderophores) in order to survive amongst 'competitors' in the environment, when the strain itself is stressed there is evidence that it degrades its own siderophores to gain nutrients. This suggestion is supported by both analytical and biochemical tests (CAS assay, TLC, HPLC, biomass measurements). A similar mechanism exists in certain Rhizobia species.

In order to determine strain 23F's ability to bind to metals other than iron (Fe III) a series of modified CAS assays were designed; the results confirmed that the isolate produces scavenging moieties responsible for the acquisition of Mn II, Co II, Cd II, Ni III, Al III, Li I, Cu II, Zn II and Mg II. There is some evidence suggesting that siderophore (catechol and hydroxamate) biosynthesis is responsible for this phenomenon.

Some interesting data reveal that in starch casein media copper sequestering by the chelating agent is not regulated by iron, which means that Fe loses its function as a repressor. This is of particular commercial value as siderophore- mediated copper decontamination could be achieved independently the presence of iron. Copper is a very toxic metal possessing a health threat to the human kind. Excessive accumulation

of the specific ion in the liver can cause chirosis, tumour, and cancer. The latter is particularly abundant in coin and wire manufacturing sites.

Moreover the organism's tolerance of different ions in terms of growth and siderophore release has also been investigated. These data encouraged the design of a model bioreactor, which has successfully demonstrated the value of 23F in terms of biodegradation and ion decontamination (Fe III, Mn II, Co II, Cd II, Ni III, Al III, Li I, Cu II, Zn II, Mg II, As II) from the environment. The system is particularly applicable for copper removal in the presence of iron supporting substantial immobilisation in minimal media.

In an attempt to investigate the molecular control of the siderophore genes in strain 23F, the information from the *S. coelicolor* sequencing project was searched. In *S. coelicolor* we have a potentially vital source of information that can be used to understand the roles of iron sequestering mechanisms. *DesA* and *desE* homologous genes were identified in *S. avermitilis* and gene specific primers were designed using the 'most' conserved areas. The amplifications were performed in both 23F and *S. coelicolor* (control), resulting in correct size products (in most of the cases), which were further sequenced.

Actinomycetes are very complex organisms that evolved intricate molecular mechanisms in order to survive and compete in their natural environment. These data could be a valuable tool that will enable us to gain an understanding of the molecular control of the siderophore genes in actinomycetes.

Chapter 5

Further work

- The physiological regulation of *desA* and *desD* genes requires further study. The role of ions on the control of this pathway can now be investigated using a blend of strictly controlled environmental conditions created using continuous culture techniques and RT PCR (Hoskisson & Hobbs, 2005).
- A useful approach would be to conduct a flux analysis given that precursor feeding appears to enhance product formation. This area requires further investigation in order to unfold specific details about the siderophore control system
- In addition to this approach the effect of disrupting the transcriptional repressor (s) DmdR should be examined, by PCR directed mutagenesis, a patented technique that has previously been employed (Gust *et al.*, 2002). Successful disruption of this gene is likely to result in a phenotype that expresses the hydroxamate in the presence of excess iron, the co-repressor of the desferrioxamine's (E) biosynthetic pathway. Biotechnologically this will be extremely valuable.
  However this is a very complex study. In the case of *S. coelicolor* it is already known that the organism possess an 'advanced' siderophore regulatory mechanism that involves at least two *dmdR*s with different iron affinities and up to ten iron boxes. Double mutations might be required and it is not clear if such an attempt will lead to a deleterious disruption. The mechanism is not well understood by the scientific community and further work is likely to unfold important information towards the understanding of the dmdR directed system.
- Radiolabelled amino acids (lysine, ornithine, serine, glutamic and aspartic acids) are required in order to determine the supplements' exact role in the biosynthetic pathway of desferrioxamine E. Such evidence should reveal whether they are directly involved in siderophore production or indirectly participate as precursors due to transamination/dehydration reactions.
- The iron-tolerance of the 23F's catecholic compound might indicate a similar mechanism to that of *M. tuberculosis*', a producer of the intracellular mycobactin and the extracellular exochelin. The former serves as a temporary iron storage system that is readily available to the organism prior to the latter's biosynthesis. To our knowledge there is no evidence of an iron storage system in streptomycetes, such as ferritin or bacterioferritin, and further investigation

is needed to reveal the fate of 23F's iron bound catechol. Sensitive X-ray analysis and dot mapping experiments would facilitate the localization of the metals and could possibly identify any potential specific metal transportation or storage mechanism.

- Further work should be devoted to the isolate 29C in order to discover the appropriate growth requirements. The media design would benefit from data generated from the Biolog Microplates. The strain overproduced a catecholic compound that scavenged iron rapidly from the environment forming strong and extremely stable ferric complexes.

Chapter 6

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7. 0. Appendices

7. 1. 0. Appendix I

23F sequence (1408 nts), GenBank accession number EF585403:

TTCGGTGGGGATTAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCC
CTGCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATACTGAG
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CGCGGCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGACG
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AGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAA
CCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGC
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31B sequence (1435 nts), GenBank accession number EF585404:

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GGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAA
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31C sequence (1215 nts), GenBank accession number EF585405:

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ACGGGGTCTAATACCGGATACGACTGCGGGAGGCATCTCCTGTGGTGGAA
AGCTCCGGCGGTGAAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGGGG
TAATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCG
GCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGT
GGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGA
GGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAG
AGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCG
CGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAG
CTCGTAGGCGGCTTGTCACGTCGGATGTGAAAGCCCGAGGCTTAACCTCG
GGTCTGCATTCGATACGGGCTGGCTAGAGTGTGGTAGGGGAGATCGGAAT
TCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCG
AAGGCGGATCTCTGGGCCATTACTGACGCTGAGGAGCGAAAGCGTGGGG
AGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGTTGGGAAC
TAGGTGTTGGCGACATTCCACGTCGTCGGTGCCGCAGCTAACGCATTAAG
TTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGAC
GGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCG
AAGAACCTTACCAAGGCTTGACATATACCGGAAAGCATTAGAGATAGTGC
CCCCCTTGTGGTCGGTATACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTC
GTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCTGTGTTG
CCAGCATGCCCTTCGGGGTGATGGGGACTCACAGGAGACCGCCGGGGTCA
ACTCGGAGGAAGGTGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGG
GACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTAC
AATGGCCGGTACAATGAG

33D sequence (1226 nts), GenBank accession number EF585406:

GCTCTGCGGGGGTCGAAAGCTCCGGCGGTGAAGGATGAGCCCGCGGCCTA
TCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGG
CCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCT
ACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGC
AGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAG
CAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAAC
TACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAAT
TATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCACGTCGGGTGTGAAAGC
CCGGGGCTTAACCCCGGGTCTGCATTCGATACGGGCAGGCTAGAGTGTGG
TAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGG
AGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCATTACTGACGCTGAGG
AGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGC
CGTAAACGGTGGGAACTAGGTGTTGGCGACATTCCACGTCGTCGGTGCCG
CAGCTAACGCATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAA
ACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGCGGAGCATGTGGCTT
AATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAA
CGTCTGGAGACAGGCGCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGC
TGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGC
AACCCTTGTCCTGTGTTGCCAGCATGCCCTTCGGGGTGATGGGGACTCACA
GGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCA
TCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAAT
GAGCTGCGATACCGTGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTT
CGGATTGGGGTCTGCAACTCGACCCCATNAAGTCGGAGTTGCTAGTAATC
GCAGATCAGCATTGCTGCGGTGAATACGTTACCCGGGCCTTGTACACACC
GCCCGTCACGTCACGAAAGTTGGTAACAC

29C sequence (997 nts), GenBank accession number EF585407:

CCGGTACACTGCGGGAGGCATCTCCTGTGGTGGAAAGCTCCGGCGGTGAA
GGATGAGCCCGCGGCCTATCAGCTTGTTGGTGGGGTAATGGCCTACCAAG
GCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTG
AGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACA
ATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCG
GGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAGAGTGACGGTACCTGCA
GAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGG
CGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTG
TCACGTCGGATGTGAAAGCCCGAGGCTTAACCTCGGGTCTGCATTCGATA
CGGGCTGGCTAGAGTGTGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGT
GAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTG
GGCCATTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTA
GATACCCTGGTAGTCCACGCCGTAAACGTTGGGAACTAGGTGTTGGCGAC
ATTCCACGTCGTCGGTGCCGCAGCTAACGCATTAAGTTCCCCGCCTGGGG
AGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACA
AGCGGCGGAGCATGTGGCTTAATTCGACGCAACTCGAAGAACCTTACCAA
GGCTTGACATATACCGGAAAGCATTAGAGATAGTGCCCCCCTTGTGGTCG
GTATACAGGTGGTGCATGGCTGTcGTCAGCTCGTGTCGTGAGATGTTGGTG
TTAAGTCCCGCAACGAGCGCAACCCTTGTCCTGTGTTGCCAGCATGCCCTT
CGGGGTGATGGGGACTCACAGGAGACCGCCGGGGTCAACTCGGAGGAAG
GT

7. 2. 0. Appendix II

GP2 MicroPlate™

A1 Water	A2 α-Cyclodextrin	A3 β-Cyclodextrin	A4 Dextrin	A5 Glycogen	A6 Inulin	A7 Mannan	A8 Tween 40	A9 Tween 80	A10 N-Acetyl-D-Glucosamine	A11 N-Acetyl-β-D-Mannosamine	A12 Amygdalin
B1 L-Arabinose	B2 D-Arabitol	B3 Arbutin	B4 D-Cellobiose	B5 D-Fructose	B6 L-Fucose	B7 D-Galactose	B8 D-Galacturonic Acid	B9 Gentiobiose	B10 D-Gluconic Acid	B11 α-D-Glucose	B12 m-Inositol
C1 α-D-Lactose	C2 Lactulose	C3 Maltose	C4 Maltotriose	C5 D-Mannitol	C6 D-Mannose	C7 D-Melezitose	C8 D-Melibiose	C9 α-Methyl-D-Galactoside	C10 β-Methyl-D-Galactoside	C11 3-Methyl Glucose	C12 α-Methyl-D-Glucoside
D1 β-Methyl-D-Glucoside	D2 α-Methyl-D-Mannoside	D3 Palatinose	D4 D-Psicose	D5 D-Raffinose	D6 L-Rhamnose	D7 D-Ribose	D8 Salicin	D9 Sedoheptulosan	D10 D-Sorbitol	D11 Stachyose	D12 Sucrose
E1 D-Tagatose	E2 D-Trehalose	E3 Turanose	E4 Xylitol	E5 D-Xylose	E6 Acetic Acid	E7 α-Hydroxybutyric Acid	E8 β-Hydroxybutyric Acid	E9 γ-Hydroxybutyric Acid	E10 p-Hydroxy-Phenylacetic Acid	E11 α-Ketoglutaric Acid	E12 α-Ketovaleric Acid
F1 Lactamide	F2 D-Lactic Acid Methyl Ester	F3 L-Lactic Acid	F4 D-Malic Acid	F5 L-Malic Acid	F6 Pyruvatic Acid Methyl Ester	F7 Succinic Acid Mono-methyl Ester	F8 Propionic Acid	F9 Pyruvic Acid	F10 Succinamic Acid	F11 Succinic Acid	F12 N-Acetyl-L-Glutamic Acid
G1 L-Alaninamide	G2 D-Alanine	G3 L-Alanine	G4 L-Alanyl-Glycine	G5 L-Asparagine	G6 L-Glutamic Acid	G7 Glycyl-L-Glutamic Acid	G8 L-Pyroglutamic Acid	G9 L-Serine	G10 Putrescine	G11 2,3-Butanediol	G12 Glycerol
H1 Adenosine	H2 2'-Deoxy Adenosine	H3 Inosine	H4 Thymidine	H5 Uridine	H6 Adenosine-5'-Monophosphate	H7 Thymidine-5'-Monophosphate	H8 Uridine-5'-Monophosphate	H9 D-Fructose-6-Phosphate	H10 α-D-Glucose-1-Phosphate	H11 D-Glucose-6-Phosphate	H12 D-L-α-Glycerol Phosphate

Carbon sources in GP2 MicroPlate.

7. 3. 0. Appendix III

Pairwise alignments of *DesA* and *DesD* of *S. coelicolor* and *S. avermitilis* (BLAST search of NCBI).

NCBI **BLAST Search Results** BLAST Entrez ?

BLASTN 2.2.11 [Jun-05-2005]

Reference:
Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schäffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.

Database: All GenBank+EMBL+DDBJ+PDB sequences (but no EST, STS, GSS,environmental samples or phase 0, 1 or 2 HTGS sequences)
3,646,835 sequences; 17,384,620,163 total letters

Query= *Des A, S. coelicolor*
(1443 letters)

>dbj|AB070936.1| *Streptomyces avermitilis* siderophore biosynthetic gene cluster
Length = 10056

Score = 1742 bits (879), Expect = 0.0
Identities = 1302/1443 (90%)
Strand = Plus / Minus

```
Query: 1    atgcgctcgcacctgctcaacgacaccaccgcggagcagtaccgccgttccgtgaccgaa 60
            ||||||||||||||||||| ||||| |||||||| | ||||||||||||||||||||||
Sbjct: 6627 atgcgctcgcacctgctcaatgacacgaccgcggatcggtaccgccgttccgtgaccgaa 6568
```

```
Query: 61   ggagtcgagcgggtggccgccaaactcgccaccaccgaccgcccgttcaccggcgtcacg 120
            ||| | ||||||||||| |||||||||||||||| |   |||||||||||||| |||||
Sbjct: 6567 ggaatagagcgggtggcggccaaactcgccaccacagcaagcccgttcaccggcatcacg 6508


Query: 121  gtcgacgccctctccccccgcatcgacgcgatcgacctcgacgagccgctgcacgacacc 180
            |||||||||||| ||||||| |||||   |||||||||||||   ||||||||||||||
Sbjct: 6507 gtcgacgccctcgcccccccggatcgagcggatcgacctcgaccgcccgctgcacgacacc 6448


Query: 181  gccgcggtcctcgacgagctggaggacgtctacctccgcgacgccgtctacttccaccac 240
             |||||||||||||||||||||| || ||||||||||||||||||||||||||||||||
Sbjct: 6447 accgcggtcctcgacgagctggaagaggtctacctccgcgacgccgtctacttccaccac 6388


Query: 241  ccccgctacctcgcccacctcaactgcccggtcgtcataccggcgctgctcggtgaagcg 300
            || |||||||||| |||||||||||||||| ||||| || ||| |  |||| || |||
Sbjct: 6387 ccgcgctacctcgcgcacctcaactgcccggtggtcatccccgcggtcgtcggcgaggcg 6328


Query: 301  gtcctgtccgccgtcaactcctccctggacacctgggaccagtcggccggcggcacgctc 360
             |||| |||||||||||||||||| |||||||||||||||||| |||||||| ||
Sbjct: 6327 atcctctccgccgtcaactcctccctcgacacctgggaccagtcggcgggcggcaccctg 6268


Query: 361  atcgagcggaagctgatcgactggacctgcgcccgcatcggcctcggcccggcggccgac 420
            |||||||| ||||| |||||||||||  ||  |||||||||||||||| || || |||
Sbjct: 6267 atcgagcgcaagctcatcgactggaccaacgagcgcatcggcctcggccccgccgcggac 6208


Query: 421  ggcgtgttcacctccggcggcacccagtccaacctccaggcgctgctcctcgcccgcgag 480
            ||||||||||||  |||||||| ||||||||||||||||||||||||||||||||||||
Sbjct: 6207 ggcgtgttcaccagcggcggcagccagtccaacctccaggcgctgctcctcgcccgcgag 6148
```

```
Query: 481  gaggcgaaggccgaggacttcgccgacctgcggatcttcgcctccgaggccagccacttc 540
            ||||| ||| |||| ||| |||||   |||||  ||||||||||||||| ||||||||||
Sbjct: 6147 gaggccaagtccgacgacctcgccagactgcgtgtcttcgcctccgaggtcagccacttc 6088


Query: 541  agcgtcaggaagtccgcgaaactgctcggcctcggccccgacgccgtcgtgtcgatcccg 600
            ||||| | |||||| |||||||||| |||||  || | ||| | ||||| ||||||||
Sbjct: 6087 agcgtgaagaagtcggcgaaactgctgggcctgagcgcggactcggtcgtctcgatcccc 6028


Query: 601  gtcgaccgcgacaagcggatgcagaccgtcgccctcgcccgcgagctggagcgctgcgcg 660
            ||||||| ||||||||| |||||||| ||||| ||||||| ||||||||| ||||||  |
Sbjct: 6027 gtcgaccacgacaagcgcatgcagacggtcgcgctcgcccacgagctggatcgctgcaag 5968


Query: 661  cgggacggcctggtccccatggccgtcgtcgccaccggcggcaccaccgacttcggctcg 720
            || ||||||||| ||||||||||||||||||||||| |||||||||||||||||||||
Sbjct: 5967 cgcgacggcctgctccccatggccgtcgtcgccaccgccggcaccaccgacttcggctcc 5908


Query: 721  atcgacccgctgccggagatcgccgggctgtgcgagcagtacggcgtgtggatgcacgtc 780
            |||||||||||||| ||||| |||| |||||||| |||| |||||  ||||||||||||
Sbjct: 5907 atcgacccgctgcccgagatagccgagctgtgcgcccagttcggcgcctggatgcacgtc 5848


Query: 781  gacgcggcctacggctgcgggctgctcgcctccctgaagtaccgggaccgcatcaccggc 840
            |||||||||||||||||||||||||| |||||||||| |||||||||| |||  ||||
Sbjct: 5847 gacgcggcctacggctgcgggctgctcacctccctgaagcaccgggaccggatcgacggc 5788


Query: 841  atcgagcgggccgactcggtcaccgtggactaccacaagtccttcttccagccggtgagt 900
            |||||||| |||||||||||||| ||||||||||||||||||||||||||||||||||
Sbjct: 5787 atcgagcgcgccgactcggtcaccgtcgactaccacaagtccttcttccagccggtgagt 5728
```

```
Query: 901  tcgtcggccgtgctggtccgggacgcggccaccctgcgccacgccacctaccacgcggag 960
            ||||| ||||||||||||||||||||  ||||||||||||||||||||||||||||||
Sbjct: 5727 tcgtccgccgtgctggtccgggacgcctccaccctgcgccacgccacctaccacgcggag 5668


Query: 961  tacctcaacccgcgccgcatggtgcaggaacgtatccccaaccaggtggacaagtccctc 1020
            ||||||||||||||||||||||  |||| || |||||||||||||| ||||||||||||
Sbjct: 5667 tacctcaacccgcgccgcatggtcgaggagcgcatccccaaccaggtcgacaagtccctc 5608


Query: 1021 cagaccacccgccgcttcgacgcgctcaagctgtggatgacgctgcgcgtgatgggcgcc 1080
            |||||||| || ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 5607 cagaccactcgtcgcttcgacgcgctcaagctgtggatgacgctgcgcgtgatgggcgcc 5548


Query: 1081 gacggcatcggcgtcctcttcgacgaggtgtgcgacctggccgccgagggctggaaactg 1140
            ||||||||||||   |||||||||||||| |||||||||| ||||||||||||||||||
Sbjct: 5547 gacggcatcggccaactcttcgacgaggtctgcgacctggcggccgagggctggaaactg 5488


Query: 1141 ctcgccgccgacccgcgcttcgacgtcgtggtccagccgtcgctgtccacgctggtcttc 1200
            |||||||||||||||||||||||||| || ||  ||||||| |||||||| || ||||||
Sbjct: 5487 ctcgccgccgacccgcgcttcgacgtggtcgtggagccgtccctgtccaccctcgtcttc 5428


Query: 1201 cgccacatcccggcggacgtcaccgatcccgccgagatcgaccgcgccaacctgtacgcc 1260
            ||| |||||||||||| ||||||||| || |||||||||| ||||||||||||||||||
Sbjct: 5427 cgctacatcccggcggccgtcaccgacccggccgagatcgatcgcgccaacctgtacgcc 5368


Query: 1261 cgcaaggccctgttcgcgtccggcgacgccgtggtcgcgggcaccaaggtcgccggtcgc 1320
            ||||||||| | ||||| |||||||||||||| |||||||||||||||||| ||| |||
Sbjct: 5367 cgcaaggccatcttcgcctccggcgacgccgtcgtcgcgggcaccaaggtcggcggccgc 5308
```

```
Query: 1321 cactacctgaagttcaccctgctcaaccccgagacgaccccggccgacatcgccgccgtc 1380
            ||||||||||||||||||||||||||||||||||| ||  ||| ||||||| |||||||
Sbjct: 5307 cactacctgaagttcaccctgctcaaccccgagaccacggcggacgacatcaccgccgtg 5248


Query: 1381 ctcgacctgatcgccggccacgccgagcagtacctgggagactcccttgaccgcgcttcc 1440
            ||||| ||||||||||||||||||||||||||||||||||| ||||||||||||||||
Sbjct: 5247 ctcgatctgatcgccggccacgccgagcagtacctgggagaatcccttgaccgcgcttcc 5188


Query: 1441 tga 1443
            |||
Sbjct: 5187 tga 5185
```

NCBI **BLAST Search Results** BLAST Entrez ?

BLASTN 2.2.11 [Jun-05-2005]

Reference:
Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schäffer,
Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997),
"Gapped BLAST and PSI-BLAST: a new generation of protein database search
programs", Nucleic Acids Res. 25:3389-3402.

Database: All GenBank+EMBL+DDBJ+PDB sequences (but no EST, STS,
GSS,environmental samples or phase 0, 1 or 2 HTGS sequences)
3,646,835 sequences; 17,384,620,163 total letters

Query= *Des D, S. coelicolor*
(1788 letters)
>dbj|BA000030.2| *Streptomyces avermitilis* MA-4680 genomic DNA, complete genome
Length = 9025608

Score = 803 bits (405), Expect = 0.0
Identities = 915/1085 (84%)
Strand = Plus / Minus

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Query: 688     ggcctggacccggccgactacctgctcatcccggtccacccctggcagtggtggaacaag 747
                ||||| ||||| | ||||||||| |||||||| ||||||||||||||||||||||||||
Sbjct: 6384281 ggcctcgaccccgacgactacctcctcatccccgtccacccctggcagtggtggaacaag 6384222


Query: 748     ctcaccgtcaccttcgccgccgaggtcgcccgcgggcacctggtgtgcctgggcgagggc 807
                ||| ||||||||||||||||||||||||||   | |  || || ||||| |||||||||
Sbjct: 6384221 ctctccgtcaccttcgccgccgaggtcgcccagcgccgtctcgtctgcctcggcgagggc 6384162
```

```
Query: 808     gacgacgagtacctggcccagcagtccatccgcaccttcttcaacgcctcgcaccccggg 867
               ||||||||||||||||||||||||||||||| || |||||||||   ||   ||||| |
Sbjct: 6384161 gacgacgagtacctggcccagcagtccatccggacgttcttcaacaagtccgcccccgag 6384102


Query: 868     aagcactacgtgaagactgccctgtccgtcctcaacatgggcttcatgcgcggtctgtcg 927
               |||||||||||| ||||| || ||||||||||||||||||||||||||||||| || |||
Sbjct: 6384101 aagcactacgtcaagacggctctgtccgtcctcaacatgggcttcatgcgcggactctcg 6384042


Query: 928     gcggcgtacatggaggccactccggccatcaacgactggctcgcccggctgatcgagggc 987
               || || |||||||||||| || |||||||||||||||||| |||| |||  ||||   |
Sbjct: 6384041 gccgcctacatggaggcgaccccggccatcaacgactggctggcccagctcgtcgacaac 6383982


Query: 988     gacccggtgctgaaggagacggggctgagcatcatccgggagcgggcggccgtcggctac 1047
               |||||||||||| |||   || || |||    ||||||| ||||| ||||||||||||||
Sbjct: 6383981 gacccggtgctcaagtccaccggcctgtcggtcatccgcgagcgcgcggccgtcggctat 6383922
```

```
Query: 1048    cggcacctggagtacgagcaggccaccgaccgctactcgccctaccgcaagatgctggcg 1107
               || ||||||||||||||   ||||||||||||||||| || |||||||||||||| ||
Sbjct: 6383921 cgccacctggagtacgaggccgccaccgaccgctactccccgtaccgcaagatgctcgcc 6383862


Query: 1108    gcgctgtggcgggagagcccggtgccgtccatccgggagggcgagacgctcgccaccatg 1167
               || |||||||| |||||||| |||||  |  ||| |||||||||||  || |||||||||
Sbjct: 6383861 gccctgtggcgcgagagccccgtgcccgcgctccaggagggcgagagcctggccaccatg 6383802


Query: 1168    gcctccctggtccaccaggaccacgagggcgcctccttcgcgggcgcgctgatcgagcgg 1227
               |||||||| |||||    ||||||| ||||||||||||||||||||||||||||||||
Sbjct: 6383801 gcctccctcgtccatgtcgaccacgcgggcgcctccttcgcgggcgcgctgatcgagcgt 6383742
```

```
Query: 1228    tccggactcacgcccaccgagtggctgcggcactacctgcgggcctactacgtcccgctg 1287
               || || ||  ||||    |||||||||||| | ||| ||||| ||||||| |   ||||||
Sbjct: 6383741 tcggggctgtcgccggtggagtggctgcgccgctatctgcgcgcctacttcacgccgctg 6383682


Query: 1288    ctgcacagcttctacgcctacgacctggtgtacatgccgcacggcgagaacgtgatcctg 1347
               |||||||||||||||| |||||||| |||||||||||||||||||||||| |||||
Sbjct: 6383681 ctgcacagcttctacgcgtacgacctcgtgtacatgccgcacggcgagaacgtcatcctc 6383622


Query: 1348    gtgctggcggacggggtggtgcggcgggcggtctacaaggacatcgccgaggagatcgcg 1407
               || ||   |||||| |  || | ||| ||| ||||||||||||||||||||||||||
Sbjct: 6383621 gtcctcaaggacggcgccgtacagcgcgcgatctacaaggacatcgccgaggagatcgcc 6383562


Query: 1408    gtgatggacccggacgcggtgctgccgccggaggtctcccgcatcgcggtggacgtgccg 1467
               ||  |||||||| | |||||||||||||| | ||||   || |||   || || || ||
Sbjct: 6383561 gtcctggacccgcaggcggtgctgccgcccgcggtcgagcggatccgcgtcgaggtcccc 6383502


Query: 1468    gacgacaagaagctcctgtcgatcttcacggacgtcttcgactgcttcttccgcttcctg 1527
               || ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 6383501 gaggacaagaagctcctgtcgatcttcacggacgtcttcgactgcttcttccgcttcctg 6383442


Query: 1528    gccgcgaacctggcggaggaggggatcgtcacggaggacgccttctggcggacggtcgcg 1587
               || ||  ||||||| || |||||  || ||   ||||| | ||||||||||||||||||
Sbjct: 6383441 gcggccgacctggccgacgagggcgtcctcggcgaggaggacttctggcggacggtcgcc 6383382


Query: 1588    gaggtcacccgggagtaccaggagtcggtgccggagctggccgacaagttcgagcggtac 1647
               |||  |   || ||||||||||||||  ||| || || ||||||||||||  || ||||
Sbjct: 6383381 gagtccgtacgcgagtaccaggagtcgacgcccgaactcgccgacaagttccggcagtac 6383322
```

```
Query: 1648    gacatgttcgcgcccgagttcgccctgtcctgcctcaaccggctccagctgcgcgacaac 1707
               ||||||||||||||| |||||||| |||||||||| ||||||||||||||||||||||
Sbjct: 6383321 gacatgttcgcgccggagttcgcgctgtcctgcctgaaccggctccagctgcgcgacaac 6383262


Query: 1708    cggcagatggtggacctggccgacccgtccggcgcgctccagctcgtcggcaccctgaag 1767
                ||||||||||||| ||||| || |||||||||||| || || || ||||||| |||||||
Sbjct: 6383261 aagcagatggtggatctggcggatccgtccggcgcactgcaactggtcggcaacctgaag 6383202


Query: 1768    aaccc 1772
               |||||
Sbjct: 6383201 aaccc 6383197
```

7. 3. 0. Appendix IV

Spectrum Report - Pumice Stone (PS1)

Live Time:	200.00	Count Rate:	1227	Dead Time:	14.10 %
Beam Voltage:	20.00	Beam Current:	2.00	Takeoff Angle:	30.00

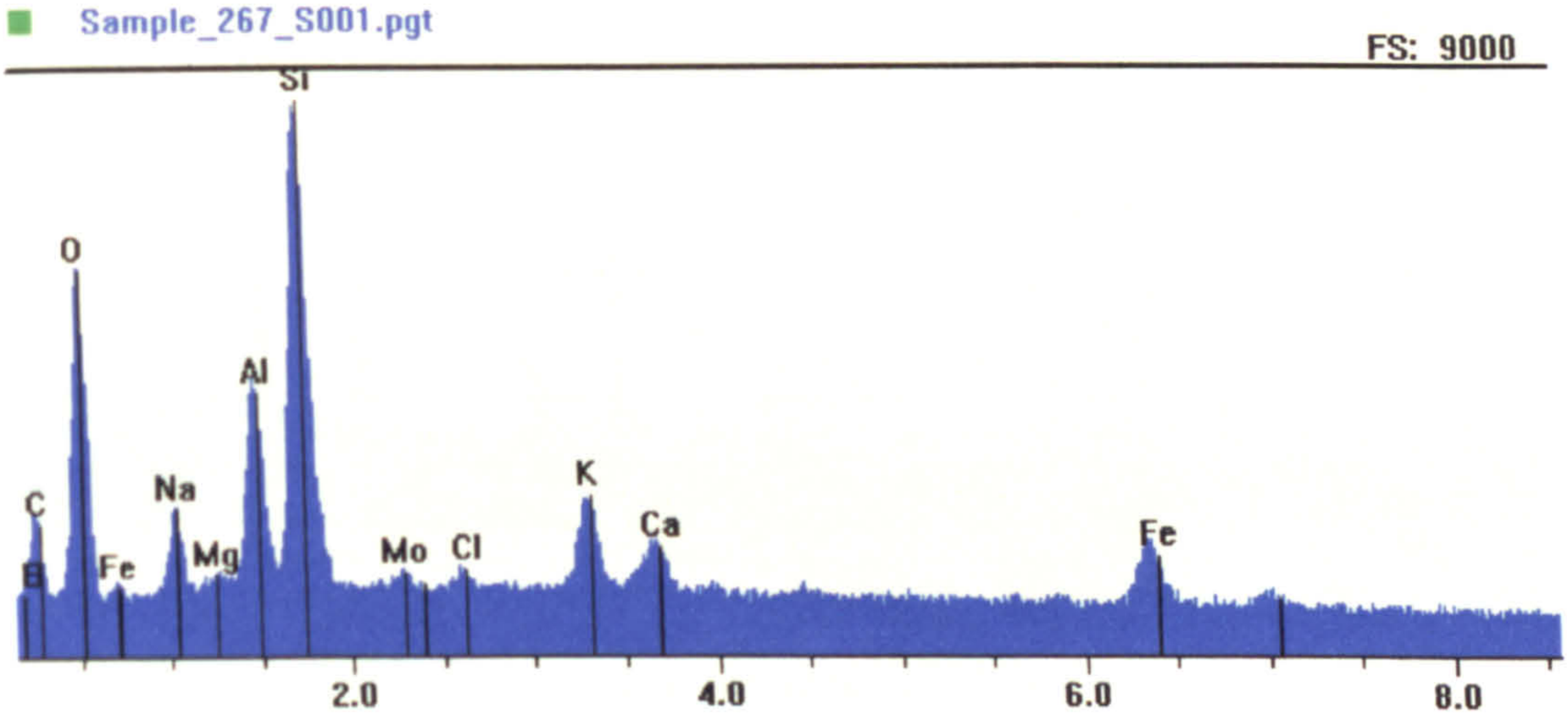

Figure 62: Elemental analysis of PS1, which contained the brick red pigment.

Element	Line	keV	KRatio	Wt%	At%	At Prop	ChiSquared
B	KA1	0.185	0.0060	11.60	16.22	0.0	27.62
C	KA1	0.277	0.0136	12.62	15.88	0.0	27.62
O	KA1	0.523	0.0000	67.18	63.47	0.0	41.54
Al	KA1	1.487	0.0077	1.33	0.75	0.0	54.82
Si	KA1	1.740	0.0340	5.19	2.79	0.0	54.82
Cl	KA1	2.622	0.0005	0.07	0.03	0.0	54.82
K	KA1	3.313	0.0040	0.51	0.20	0.0	5.06
Ca	KA1	3.691	0.0016	0.20	0.07	0.0	5.06
Fe	KA1	6.403	0.0039	0.48	0.13	0.0	14.49
Mo	LA1	2.293	0.0010	0.16	0.03	0.0	54.82
Mg	KA1	1.254	0.0002	0.05	0.03	0.0	54.82
Na	KA1	1.041	0.0019	0.61	0.40	0.0	54.82
Total			**0.0745**	**100.00**	**100.00**	**0.0**	**36.03**

Table 24: Elemental analysis showing the data generated for PS1, which contained the brick red pigment.

Spectrum Report - Pumice Stone (PS2)

Live Time:	200.00	Count Rate:	1321	Dead Time:	13.04 %
Beam Voltage:	20.00	Beam Current:	2.00	Takeoff Angle:	30.00

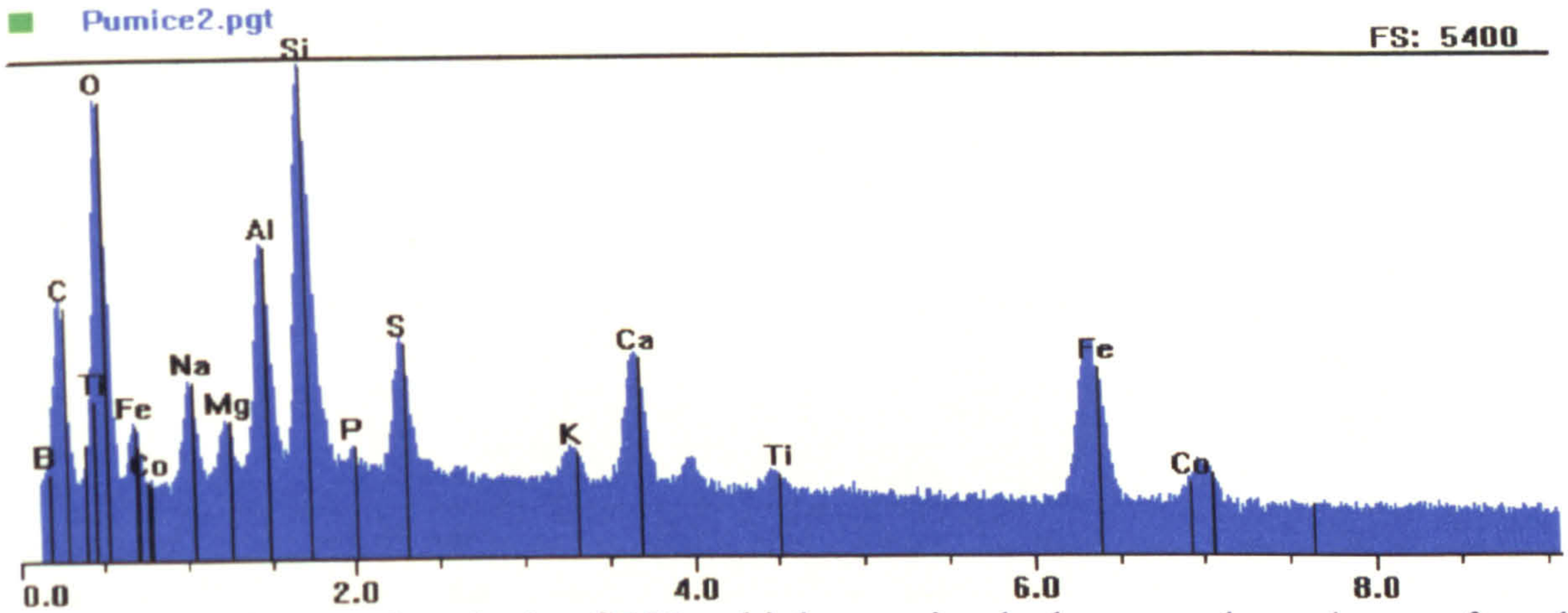

Figure 63: Elemental analysis of PS2, which contained a brown coloured stone found within the pumice.

Element	Line	keV	KRatio	Wt%	At%	At Prop	ChiSquared
B	KA1	0.185	0.0093	9.59	13.10	0.0	36.67
C	KA1	0.277	0.0314	16.91	20.80	0.0	36.67
O	KA1	0.523	0.0000	69.59	64.26	0.0	36.67
Na	KA1	1.041	0.0010	0.36	0.23	0.0	36.67
Mg	KA1	1.254	0.0003	0.08	0.05	0.0	36.67
Al	KA1	1.487	0.0033	0.63	0.34	0.0	36.67
Si	KA1	1.740	0.0090	1.41	0.74	0.0	36.67
S	KA1	2.307	0.0021	0.28	0.13	0.0	36.67
K	KA1	3.313	0.0004	0.05	0.02	0.0	15.32
Ca	KA1	3.691	0.0024	0.27	0.10	0.0	15.32
Ti	KA1	4.510	0.0002	0.03	0.01	0.0	1.10
Fe	KA1	6.403	0.0058	0.73	0.19	0.0	90.73
Co	KA1	6.929	0.0005	0.06	0.01	0.0	90.73
P	KA1	2.013	0.0001	0.01	0.01	0.0	36.67
Total			**0.0657**	**100.00**	**100.00**	**0.0**	**33.58**

Table 25: Elemental analysis showing the data generated for PS2, which contained a brown coloured stone found within the pumice.

Spectrum Report - Pumice Stone (PS3)

Live Time:	200.00	Count Rate:	3522	Dead Time:	29.17 %
Beam Voltage:	20.00	Beam Current:	2.00	Takeoff Angle:	30.00

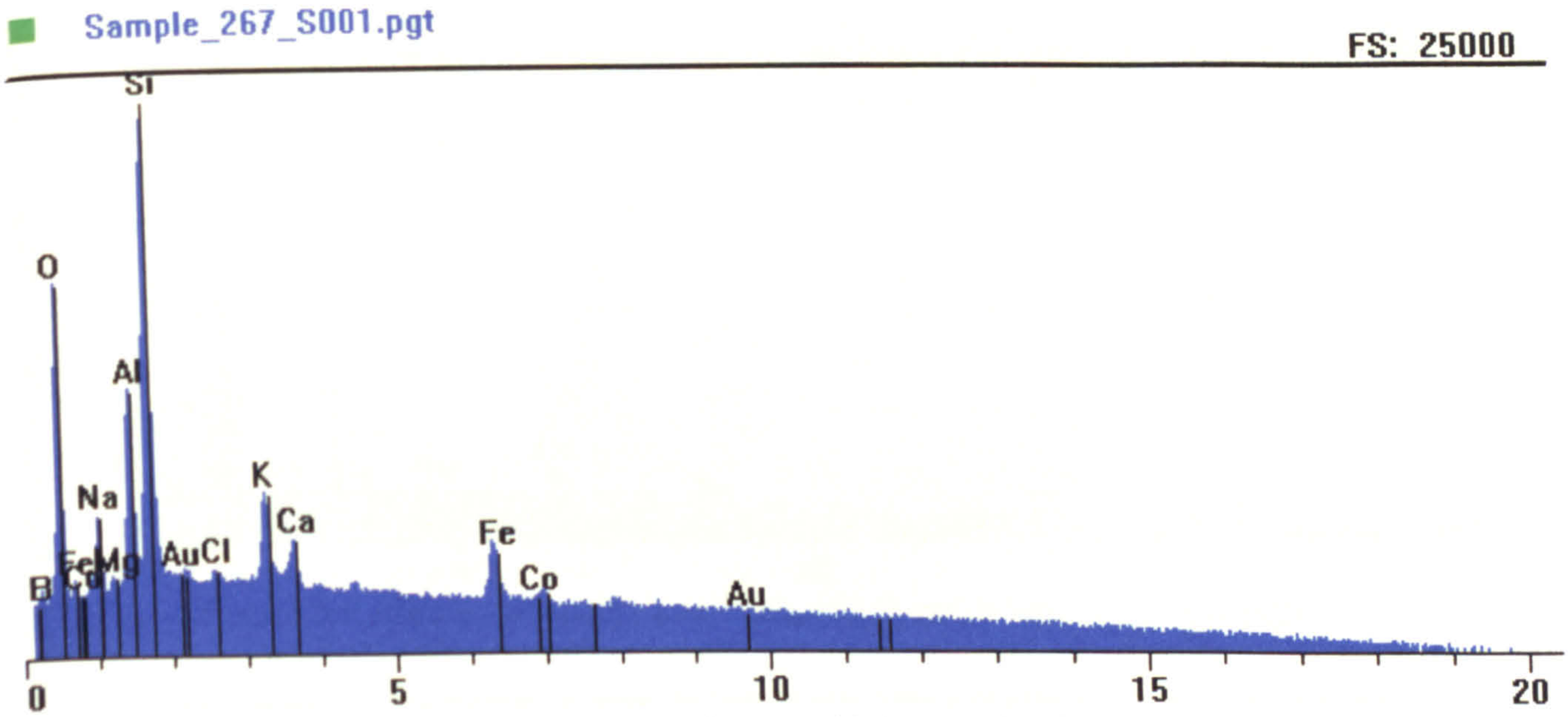

Figure 64: Elemental analysis of PS3, which contained the yellow pigment.

Element	Line	keV	KRatio	Wt%	At%	At Prop	ChiSquared
B	KA1	0.185	0.0069	18.48	27.59	0.0	35.14
O	KA1	0.523	0.0000	60.28	60.82	0.0	261.21
Na	KA1	1.041	0.0046	1.33	0.94	0.0	261.21
Al	KA1	1.487	0.0185	3.05	1.82	0.0	261.21
Si	KA1	1.740	0.0870	13.14	7.56	0.0	261.21
K	KA1	3.313	0.0110	1.42	0.58	0.0	23.98
Ca	KA1	3.691	0.0039	0.48	0.20	0.0	23.98
Fe	KA1	6.403	0.0098	1.20	0.35	0.0	31.48
Cl	KA1	2.622	0.0008	0.12	0.05	0.0	261.21
Au	MA1	2.121	0.0018	0.36	0.03	0.0	261.21
Mg	KA1	1.254	0.0003	0.07	0.04	0.0	261.21
Co	KA1	6.929	0.0006	0.08	0.02	0.0	31.48
Total			**0.1452**	**100.00**	**100.00**	**0.0**	**173.20**

Table 26: Elemental analysis showing the data generated for PS3, which contained the yellow pigment.

Spectrum Report - Pumice Stone (PS 4)

Live Time:	175.48	Count Rate:	1071	Dead Time:	10.67 %
Beam Voltage:	20.00	Beam Current:	2.00	Takeoff Angle:	30.00

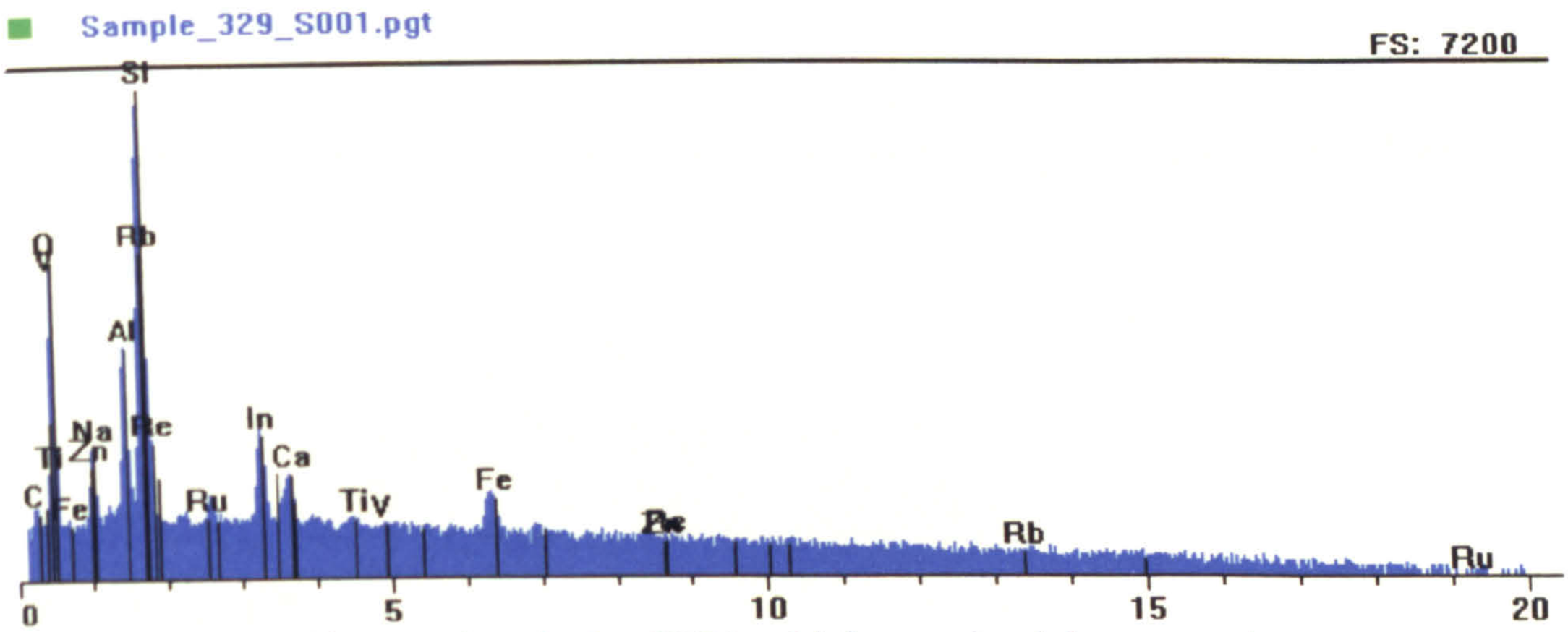

Figure 65: Elemental analysis of PS4, which contained the green pigment.

Element	Line	keV	KRatio	Wt%	At%	At Prop	ChiSquared
Rb	LA1	1.694	0.0680	7.96	2.15	0.0	16.96
Si	KA1	1.740	0.1141	12.99	10.68	0.0	16.96
V	KA1	4.951	0.0002	0.03	0.01	0.0	16.96
Ca	KA1	3.691	0.0050	0.54	0.31	0.0	16.96
Al	KA1	1.487	0.0274	3.24	2.77	0.0	16.96
Re	LA1	8.652	0.0005	0.06	0.01	0.0	1.00
In	LA1	3.286	0.0450	5.61	1.13	0.0	16.96
Fe	KA1	6.403	0.0119	1.34	0.55	0.0	7.16
Na	KA1	1.041	0.0069	1.16	1.16	0.0	2.03
C	KA1	0.277	0.0142	7.53	14.46	0.0	2.03
Ru	LA1	2.558	0.0048	0.65	0.15	0.0	16.96
O	KA1	0.523	0.0670	17.67	25.49	0.0	2.03
Ti	KA1	4.510	0.0010	0.12	0.06	0.0	16.96
Zn	KA1	8.637	0.0006	0.06	0.02	0.0	1.00
Total			**0.3667**	**58.95**	**58.95**	**0.0**	**11.79**

Table 27: Elemental analysis showing the data generated for PS4, which contained the green pigment.

Spectrum Reports - Pumice Stone (PS5)

Live Time:	200.00	Count Rate:	1287	Dead Time:	12.79 %
Beam Voltage:	20.00	Beam Current:	2.00	Takeoff Angle:	30.00

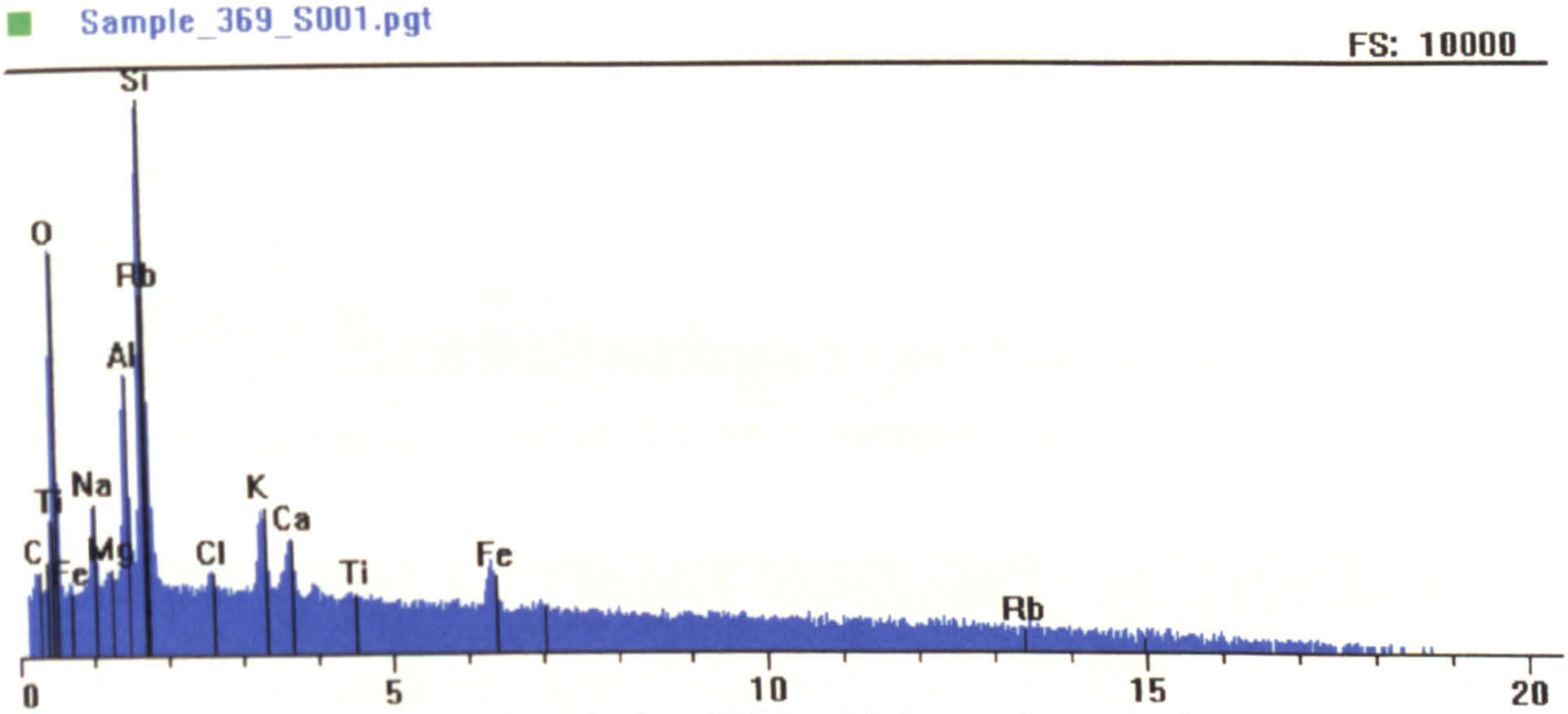

Figure 66: Elemental analysis of PS5, which was the typical grey pumice.

Element	Line	keV	KRatio	Wt%	At%	At Prop	ChiSquared
O	KA1	0.523	0.0000	56.55	64.13	0.0	11.18
Na	KA1	1.041	0.0067	1.86	1.47	0.0	19.22
Al	KA1	1.487	0.0249	3.98	2.68	0.0	19.22
Si	KA1	1.740	0.0935	13.75	8.88	0.0	19.22
K	KA1	3.313	0.0108	1.41	0.65	0.0	4.19
Ca	KA1	3.691	0.0066	0.82	0.37	0.0	4.19
Fe	KA1	6.403	0.0076	0.92	0.30	0.0	7.37
Rb	LA1	1.694	0.0476	7.27	1.54	0.0	19.22
Cl	KA1	2.622	0.0011	0.17	0.09	0.0	1.19
Mg	KA1	1.254	0.0005	0.10	0.07	0.0	19.22
C	KA1	0.277	0.0107	13.10	19.78	0.0	11.18
Ti	KA1	4.510	0.0006	0.08	0.03	0.0	1.31
Total			**0.2107**	**100.00**	**100.00**	**0.0**	**12.58**

Table 28: Elemental analysis showing the data generated for PS5, which was the typical grey pumice.

Spectrum Report - Pumice Stone (PS6)

Live Time:	200.00	Count Rate:	1195	Dead Time:	12.03 %
Beam Voltage:	20.00	Beam Current:	2.00	Takeoff Angle:	30.00

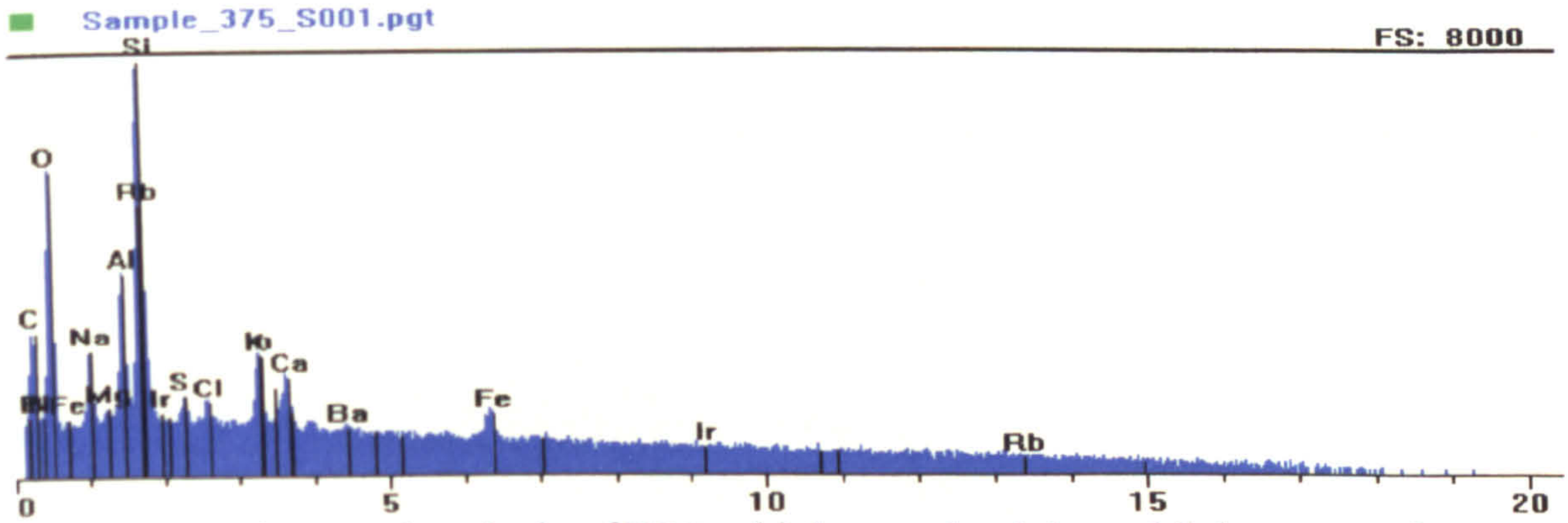

Figure 67: Elemental analysis of PS6, which contained the reddish-orange pigment.

Element	Line	keV	KRatio	Wt%	At%	At Prop	ChiSquared
B	KA1	0.185	0.0035	6.11	8.95	0.0	32.26
C	KA1	0.277	0.0149	14.14	18.64	0.0	32.26
N	KA1	0.392	0.0028	2.05	2.31	0.0	32.26
O	KA1	0.523	0.0000	65.35	64.65	0.0	32.26
Na	KA1	1.041	0.0027	0.85	0.59	0.0	14.78
Al	KA1	1.487	0.0084	1.44	0.85	0.0	14.78
Si	KA1	1.740	0.0333	4.98	2.81	0.0	14.78
S	KA1	2.307	0.0009	0.15	0.07	0.0	14.78
Cl	KA1	2.622	0.0009	0.13	0.06	0.0	14.78
K	KA1	3.313	0.0040	0.50	0.20	0.0	14.78
Ca	KA1	3.691	0.0030	0.36	0.14	0.0	14.78
Fe	KA1	6.403	0.0024	0.29	0.08	0.0	6.21
In	LA1	3.286	0.0029	0.40	0.06	0.0	14.78
Rb	LA1	1.694	0.0197	3.06	0.57	0.0	14.78
Mg	KA1	1.254	0.0001	0.02	0.02	0.0	14.78
Ir	LA1	9.175	0.0003	0.05	0.00	0.0	1.04
Ba	LA1	4.465	0.0008	0.11	0.01	0.0	14.78
Total			**0.1005**	**100.00**	**100.00**	**0.0**	**16.66**

Table 29: Elemental analysis showing the data generated for PS6, which contained the reddish-orange pigment.

Spectrum Report - Coal

Live Time:	200.00	Count Rate:	806	Dead Time:	12.63 %
Beam Voltage:	20.00	Beam Current:	2.00	Takeoff Angle:	30.00

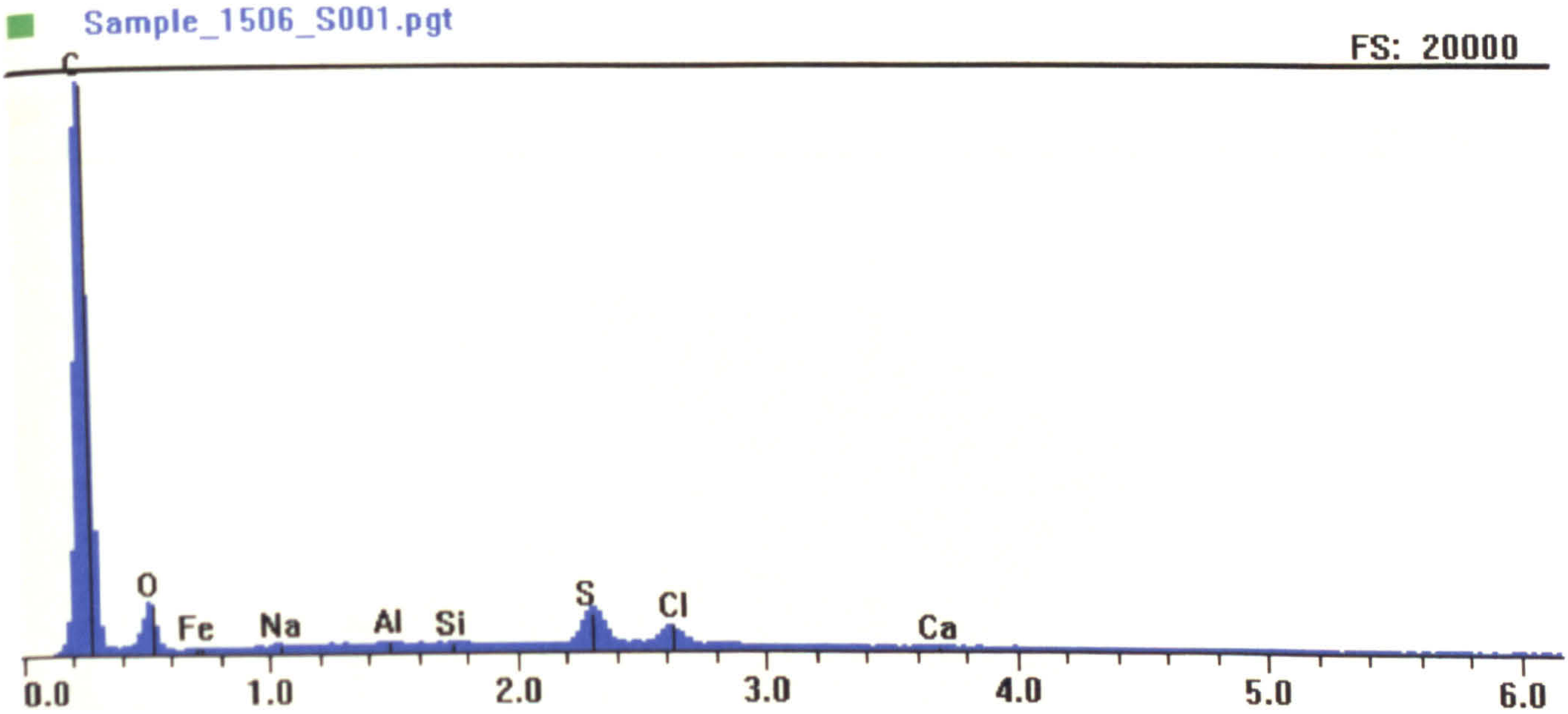

Figure 68: Elemental analysis of coal.

Element	Line	keV	KRatio	Wt%	At%	At Prop	ChiSquared
C	KA1	0.277	0.0990	26.79	32.94	0.0	2076.29
O	KA1	0.523	0.0000	72.21	66.63	0.0	27.76
Cl	KA1	2.622	0.0022	0.27	0.11	0.0	4.78
S	KA1	2.307	0.0036	0.45	0.21	0.0	4.78
Fe	KA1	6.403	0.0012	0.16	0.04	0.0	1.27
Na	KA1	1.041	0.0002	0.06	0.04	0.0	0.65
Al	KA1	1.487	0.0001	0.03	0.01	0.0	1.26
Si	KA1	1.740	0.0001	0.02	0.01	0.0	1.26
Ca	KA1	3.691	0.0001	0.01	0.00	0.0	1.41
Total			**0.1065**	**100.00**	**100.00**	**0.0**	**921.76**

Table 30: Elemental analysis showing the data generated for the coal sample.

Spectrum Report - Sponge (Sample 1)

Live Time:	51.47	Count Rate:	231	Dead Time:	4.33 %
Beam Voltage:	20.00	Beam Current:	2.00	Takeoff Angle:	30.00

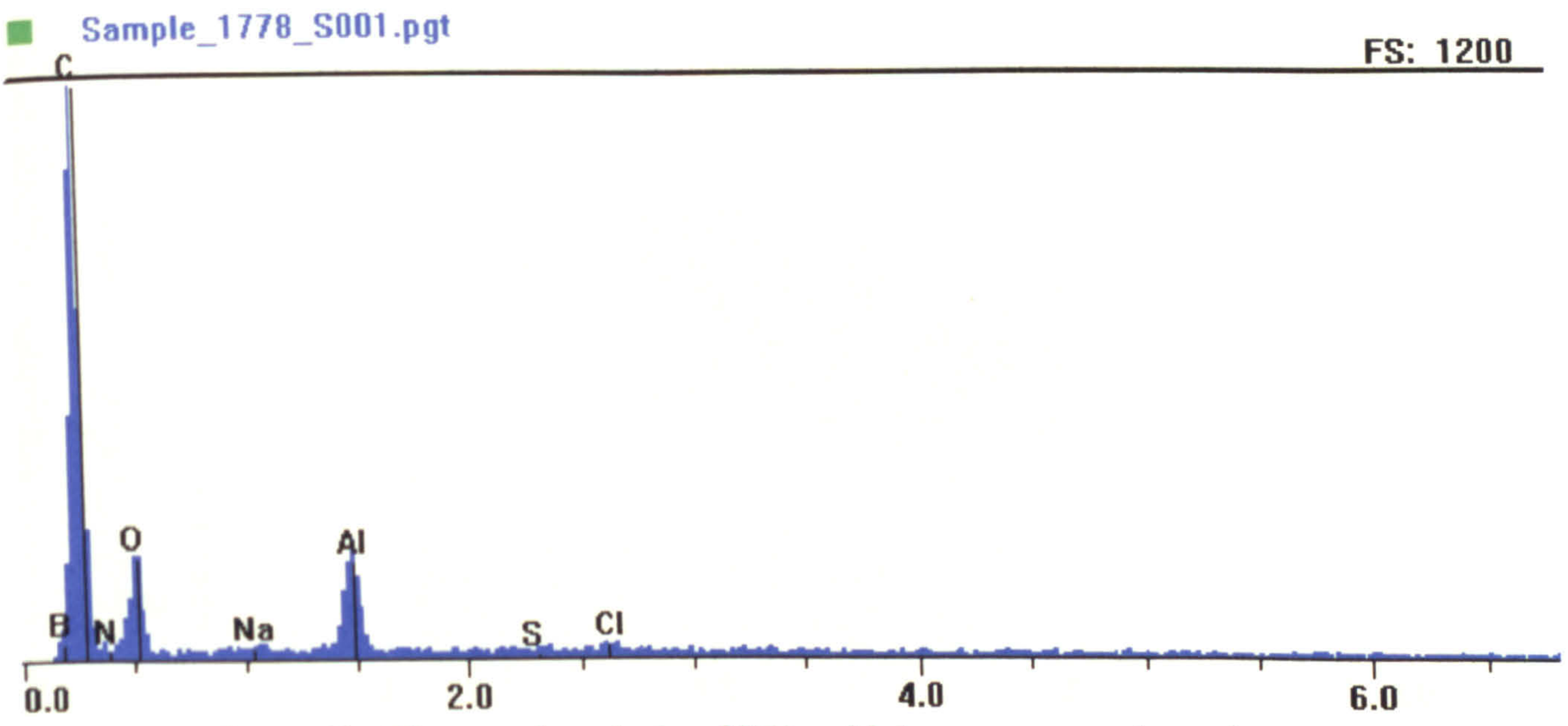

Figure 69: Elemental analysis of SP1, which was green coloured sponge.

Element	Line	keV	KRatio	Wt%	At%	At Prop	ChiSquared
B	KA1	0.185	0.0091	6.28	8.47	0.0	72.91
C	KA1	0.277	0.0655	20.87	25.33	0.0	72.91
N	KA1	0.392	0.0008	0.54	0.56	0.0	72.91
O	KA1	0.523	0.0000	71.63	65.27	0.0	72.91
S	KA1	2.307	0.0001	0.01	0.00	0.0	1.09
Cl	KA1	2.622	0.0003	0.04	0.02	0.0	1.09
Na	KA1	1.041	0.0002	0.06	0.04	0.0	0.97
Al	KA1	1.487	0.0029	0.56	0.30	0.0	0.55
Total			**0.0789**	**100.00**	**100.00**	**0.0**	**31.83**

Table 31: Elemental analysis showing the data generated for SP1, which was green coloured sponge.