CHAPTER VI

ISOLATION AND CHARACTERIZATION OF MUTANTS DEFECTIVE IN IRON TRANSPORT SYSTEM
Arceneaux and Lankford reported for the first time isolation of an induced siderophore auxotrophic mutant of *Bacillus megaterium* (1). They showed that these mutants required schizokinen for the growth. Further, it was also observed that several siderophores as well as synthetic iron / metal chelators could substitute for schizokinen requirement. The possibility that schizokinen may have a function as specific cofactor rather than iron transporting compound was ruled out since variety of chemically diverse chelating agents could substitute for schizokinen. Before this report, there was only one report regarding wild type siderophore auxotrophic strains (2). Later on reports on mutants showed important finding which led to discovery of molecular mechanisms regulating iron uptake as well as biosynthesis of siderophores (3-11).

To know the significance of siderophores to the producing organism as well as for better understanding of the iron uptake mechanism, it was pertinent to isolate mutants having defects in iron transport system. For this purpose chemical mutagenesis (N-methyl-N'-nitro-N-nitrosoguanidine) and transposon (Tn5) mutagenesis were carried out. Screening was done to isolate mutants defective in iron transport machinery. Initially, the mutagenized culture was spread on Ashby's mannitol agar (AMA), containing 100 μM iron (as FeCl₃). Well isolated colonies were grided on AMA plates containing bipyridyl (200 μM). The clones which failed to grow on bipyridyl containing medium were selected for further studies.

An alternative approach was also used where the mutagenized culture was directly spread on the chrome azurol-S containing agar (CAS-agar) plates. Those clones that failed to show orange halo around the colony or showed larger halo were selected. Overall approximately 14,000 clones were screened, out of which 8 clones showed defect in the iron transport system. From these 8 mutants, one mutant was obtained by NTG mutagenesis whereas other seven were obtained by transposon mutagenesis. Six transposon mutants initially showed decreased or no siderophore production. But these mutants on subsequent transfers in Fe-limited (without any iron chelator) medium, somehow regained the
property of siderophore production. The seventh transposon mutant \(P_{4-5}\) was siderophore overproducing (Table 1). The NTG mutant (Rhizobium GN1-M) was unable to produce any siderophore. As these later two mutants appeared to be stable they were used for the subsequent studies.

The mutant GN1-M was not able to grow on bipyridyl containing agar plates (Fig. 1) but could grow well when medium was supplemented with iron. In Fe-limited medium without any iron chelator, this mutant could not grow second transfer onwards. As this mutant could grow in Fe-limited medium in the first transfer, the culture supernatant was checked for any siderophore. We could not detect any siderophore production. The culture supernatant also failed to change the blue colour of the CAS assay solution. These results confirmed that the mutant was unable to produce any siderophore. The growth curve pattern of this mutant in Fe-limited medium showed that the mutant could regain the growth when supplemented with siderophore from the wild type (Fig. 2) suggesting that probably the mutant could use the siderophore for iron acquisition. Hence, bioassay was carried out where it was observed that GN1-M showed good growth around the well containing siderophore (Fig. 3). These results strongly indicated that GN1-M was a biosynthesis mutant i.e. it was not able to produce / synthesize siderophore but the machinery required for the transport of Fe-siderophore complex was intact. Sevinc and Page have shown that Azotobacter vinelandii strain defective in siderophore production could not grow in EDDHA containing medium when the inoculum was prepared in Fe-limited medium, but this mutant showed growth when inoculant cells were grown in normal medium (12). A. vinelandii is known to produce bacterioferritin (13, 14) which could probably act as iron reserve for the cells which were priorly grown in normal medium.

In case of mutant \(P_{4-5}\), there was decrease in growth under Fe-limited conditions (Fig. 4), but this mutant could grow in Fe-limited medium
<table>
<thead>
<tr>
<th>Organism</th>
<th>Siderophore production (%)$^+$</th>
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<tbody>
<tr>
<td>Wild type</td>
<td>100</td>
</tr>
<tr>
<td>GN1-M</td>
<td>ND**</td>
</tr>
<tr>
<td>P4-5</td>
<td>134.3</td>
</tr>
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</table>

$^*$ Cultures were grown in Ashby's mannitol broth.

$^+$ The concentration of siderophore in case of wild type (27.7 μg/ml) was considered as 100%.

$^{**}$ ND : Not detected.
Fig. 1: Comparison of growth of cowpea Rhizobium GN1 and mutant GN1-M on bipyridyl (200 μM) containing agar plates.
Fig. 2: Growth curve of cowpea Rhizobium GN1 (wild type) and its mutant GN1-M.

+ Wild type in Fe-limited AMB
▲ GN1-M in Fe-limited AMB
■ GN1-M in (Fe-limited AMB + siderophore)
● GN1-M in Fe-supplemented AMB
Figure 2:

![Graph showing growth (O.D. at 600 nm) over time (h)].

- Y-axis: Growth (O.D. at 600 nm)
- X-axis: Time (h)
- Data points at 0, 7, 14, 21, 28 hours.
Fig. 3: Bioassay of the siderophore with mutant GN1-M.

A: Well containing 100 μg siderophore
B: Well containing 1 μg siderophore
C: Control (well with sterile saline)
Fig. 4: Growth curve of cowpea *Rhizobium* GN1 (wild type) and its mutant $P_4^{-5}$.

- Wild type in Fe-limited AMB
- Mutant $P_4^{-5}$ in Fe-limited AMB
- Mutant $P_4^{-5}$ in (Fe-limited AMB + siderophore)
- Mutant $P_4^{-5}$ in Fe-supplemented AMB
Figure 4:

Growth (O.D. at 600 nm) vs. Time (h)
even after subsequent transfers. Compared to the wild type this mutant showed decreased growth in Fe-limited medium, even though it produced more siderophore than the wild type. Even when this mutant was supplemented with siderophore from the wild type, it did not show any significant increase in growth. The biological activity of the siderophore of this mutant was checked. This siderophore could promote the growth of the wild type as well as GN1-M. The CAS assay test also showed strong positive reaction (i.e. immediate colour change). Hence, the probable reason for increased siderophore production and decreased growth of the mutant could be attributed to the defective transport system. Hence, this mutant was considered as 'transport mutant'.

Gill and Neilands isolated mutants of *Rhizobium melliloti* defective in iron uptake system (15). They could classify the mutants into three categories viz. mutants defective in production of siderophore (*Rzb"*), mutants able to produce siderophore but defective in uptake (*Rbu"*) and mutants which constitutively synthesized siderophore (*Rbr"*). In case of cowpea *Rhizobium* GN1, the NTG mutant i.e. GN1-M was similar to *Rzb"* mutant since it could not synthesize siderophore.

We checked the mutants for their sensitivity and ability to sustain effect of synthetic iron chelator bipyridyl. The mutant GN1-M showed linear decrease in growth with increase in concentration of bipyridyl. There was 80% decrease in growth at 40 μM concentration (Fig. 5). Comparatively mutant P4-5 could sustain higher concentration of bipyridyl. It showed about 45% reduction in growth at 200 μM concentration whereas in case of wild type, there was only 20% reduction in growth at 200 μM concentration of bipyridyl.

When GN1-M was studied for its ability to take up 55Fe-siderophore enhanced iron uptake was observed (Fig. 6). Further, it was found that iron limited cells were efficient in iron uptake compared to iron sufficient cells, almost similar to wild type. The uptake studies with
Fig. 5: Effect of bipyridyl on growth of cowpea *Rhizobium* GN1 and its mutants. Cultures were grown in AMB (with different concentrations of bipyridyl) prepared in double glass distilled water.

- Wild type
- Mutant $P_4^{-5}$
- Mutant GN1-M

(Growth in AMB without bipyridyl was considered as 100%)
Figure 5:

Graph showing the relationship between bipyridyl concentration (pM) and % growth.

% Growth

Bipyridyl concentration (µM)
Fig. 6: Siderophore mediated iron uptake by mutant GN1-M.

○ Wild type (priorly grown in Fe-limited medium)

□ Mutant GN1-M (priorly grown in Fe-limited medium)

■ Mutant GN1-M (priorly grown in Fe-sufficient medium)
Fe Uptake (pmoles/mg protein)

Figure 6: 

$^{55}$Fe Uptake (pmoles/mg protein)

Time (min)
P$_4$-5 showed that this mutant was also capable of taking up $^{55}$Fe-siderophore (Fig. 7). But compared to wild type the uptake in this mutant appeared to be decreased. These results suggested that the inducible iron uptake system in both the mutants was intact.

Outer membrane protein profiles of both the mutants revealed 80 kDa and 76 kDa proteins under Fe-limited conditions (Fig. 8, 9). Similar two proteins were detected in the outer membrane of the Fe-limited wild type cells (Chapter IVB). This supports our earlier speculation that one of these proteins might be acting as a receptor protein for Fe-siderophore complex.

Here we could not exactly find out the reason for increased siderophore production by P$_4$-5 as it appears to take up Fe-siderophore quite well. The possible reasons for increased siderophore production could be -

i) Mutation leading to the increase in synthesis of enzyme/s involved in siderophore biosynthesis. Here it could also be possible that more amount of precursors or intermediate metabolites are channelised or made available for the biosynthesis of siderophore.

ii) Decrease in iron reductase activity which may affect release of iron from Fe-siderophore complex resulting in pseudo deficiency of iron and thus increased siderophore production.

iii) Since the mutant was less efficient than the wild type in iron uptake, it could be possible that there was something wrong with the interaction between receptor and the Fe-siderophore complex.

In order to investigate later two possibilities experiments were carried out. We studied iron reductase activity in vitro. In both the mutants
Fig. 7: Siderophore mediated iron uptake by mutant $P_{4-5}$.

- Wild type (priorly grown in Fe-limited medium)
- Mutant $P_{4-5}$ (priorly grown in Fe-limited medium)
- Mutant $P_{4-5}$ (priorly grown in Fe-sufficient medium)
Figure 7:

[Graph showing uptake (pmoles/mg protein) over time (min)]
Fig. 8: Outer membrane protein (OMP) profile of cowpea *Rhizobium* GN1 (wild type) and its mutant GN1-M.

Lane 1 & 2: OMPs of mutant GN1-M grown in Fe-limited medium.
Lane 3: OMPs of mutant GN1-M grown in Fe-sufficient medium.
Lane 4: OMPs of wild type grown in Fe-limited medium.
Lane 5: OMPs of wild type grown in Fe-sufficient medium.

(Arrows indicate iron repressible outer membrane proteins)
Fig. 9: Outer membrane protein (OMP) profile of mutant P₄⁻⁵.

Lane 1: OMPs of mutant P₄⁻⁵ grown in Fe-sufficient medium.

Lane 2: OMPs of mutant P₄⁻⁵ grown in Fe-limited medium.

(Arrows indicate iron repressible outer membrane proteins)
i.e. P_4^-5 and GN1-M, the iron reductase activity was unaffected and it was comparable with that in the wild type (Table 2). This suggested that release of iron from the complex was not a growth limiting factor for the mutants. And probably this could not be the reason for the increased siderophore production in mutant P_4^-5.

In another experiment we carried out uptake studies using different concentrations of $^{55}$Fe-siderophore complex. It was observed that in case of mutant P_4^-5 the saturation of uptake was obtained at 1 μM concentration (Fig. 10) whereas in case of wild type strain, it was obtained at 0.5 μM concentration of $^{55}$Fe-siderophore complex (Chapter IVA). Lineweaver-Burk plot showed that the Km value for the $^{55}$Fe-siderophore complex in P_4^-5 mutant was 0.25 μM (Fig. 11), whereas in case of the wild type it was 0.105 μM (Chapter IVA). From these results it appears that probably the affinity of the receptor for $^{55}$Fe-siderophore complex is reduced in case of mutant P_4^-5. Hence, the rate at which the siderophore can supply iron to the mutant would be lower as compared to the wild type. This could be the possible reason for the decreased growth and increased siderophore production by the mutant P_4^-5. Studies in Pseudomonas have shown that the interaction between the Fe-siderophore complex and the receptor is not only crucial but a complex phenomenon (16, 17). Bitter et al. isolated a transposon mutant of Pseudomonas putida WCS-358 which lack a specific receptor for pseudobactin 358 (one of the pseudobactin siderophores produced by Pseudomonas putida) (16). This mutant could take up iron from different pseudobactins similar to the wild type. But with pseudobactin 358, the uptake of iron was only 30% compared to the wild type. Since this mutant does not have a receptor for this particular pseudobactin it may have some alternate pathway for whatever iron taken up (30%) from pseudobactin 358. The results have also shown that the extent and rate of iron uptake with different pseudobactins were different. Marugg et al. showed that Pseudomonas putida WCS-374, which produced pseudobactin
Table 2: Iron reductase activity in cowpea *Rhizobium* GN1 and its mutants:

<table>
<thead>
<tr>
<th>Culture</th>
<th>Iron reductase activity[^1]</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>[nmoles of Fe(II) formed min(^{-1}) mg protein(^{-1})]</td>
</tr>
<tr>
<td>Wild type</td>
<td>22.8</td>
</tr>
<tr>
<td>GN1-M</td>
<td>18.4</td>
</tr>
<tr>
<td>P4-5</td>
<td>21.3</td>
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[^1]: Fe-siderophore complex was used as substrate in the assay system.
Fig. 10: Relative rate of $^{55}$Fe uptake in mutant $P_{4-5}$ as a function of the $^{55}$Fe-siderophore concentration.
Figure 10:

The graph shows the relationship between the uptake rate (pmoles/mg protein/min) and the $^{55}$Fe-siderophore concentration (pM). The x-axis represents the $^{55}$Fe-siderophore concentration (µM) ranging from 0.5 to 5.0, while the y-axis represents the uptake rate ranging from 0 to 20 pmoles/mg protein/min. The data points are connected by a curve, indicating a non-linear relationship between the two variables.
Fig. 11: Lineweaver-Burk's plot of the inverse of uptake rate (1/R) versus the inverse of \(\frac{1}{[S]}\) in mutant \(P_{4-5}\).
374 was inhibited by pseudobactin 358 (17). This showed that P. putida WCS-374 neither have a receptor for iron complex of pseudobactin 358 nor the receptor for pseudobactin 374 can promote iron uptake mediated by pseudobactin 358.

Thus the results presented here are indicative of the possible involvement of siderophores for growth under Fe-limited conditions. Alongwith production of siderophore, intactness of iron uptake system i.e. receptor proteins and the enzyme machinery required for iron release are essential features for the better survival of the organism under Fe-limitation.
REFERENCES


