Siderophore-mediated iron uptake in mycobacteria

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Introduction: Iron is vital for the survival and proliferation of microorganisms, while mycobacteria also require this element for their survival within the host. To meet the demand for iron, mycobacteria synthesise and utilise specific high-affinity iron-binding compounds (siderophores) which help them grow in the' iron-restricted conditions of the host [1, 2] and also participate in the uptake of iron across the thick lipid cell wall [3].

Two types of siderophores are produced by mycobacteria [3]. Exochelin occurs extracellularly to act as a scavenger, and mycobactin occurs on the cell wall to act as a transporter. Specific membrane proteins are also produced by several mycobacteria for the transport of siderophore-ferric iron complexes [4].

Recent work [5] demonstrated that the growth of *Mycobacterium tuberculosis* strains was increased with increasing concentrations of iron in the medium and that the concentrations of exochelins and mycobactins, which are highest under iron-deficient conditions, registered marked decreases. It does not follow, however, that exochelins are involved in the uptake of iron by mycobacteria.

We have therefore studied the uptake of iron by four strains of mycobacteria in the absence and in the presence of exochelins released by these strains.

Materials and methods: Four different strains of mycobacteria – a saprophyte, *M. smegmatis* (TMC 1515); an avirulent strain $H_{37}R_a$ (TMC 201); a standard virulent strain $H_{37}R_v$ (TMC 102) and a South Indian low virulent (SILV) strain of *M. tuberculosis* – were kindly supplied by the Bacteriology Department of the Tuberculosis Research Centre, Madras, India. Strains were grown on Lowenstein-Jensen (LJ) slopes to their log phase and were inoculated into a synthetic medium (15 mL), which was prepared in iron-free glassware [6]. with 0.01 mL suspension of 3 X 10^6 cells mL^{-1} of *M. smegmatis*, 9 x 10^5 cells mL^{-1} for $H_{37}R_a$, 1 x 10^6 cells mL^{-1} for $H_{37}R_v$ and 9 x 10^5 cells mL^{-1} for SILV.

Five sets of flasks (each containing 15 µg Fe^{2+} mL^{-1}) were set up for each strain. One was used as a control and to the other four sets were added various concentrations (1.25,2.5, 5 and 10 µg mL^{-1}) of exochelins (isolated from the same strain [7] used for inoculation). After inoculation, the flasks were incubated at 37°C without shaking. The experiment was set

Table I: Effect of addition of increasing concentrations of exochelins on iron uptake by M. smegmatis *at different days (means ± SD of 4 estimates).*

Exochelin concentration (µg mL^{-1})	Iron uptake (µg mL^{-1}) at the following days after inoculation			
	2	4	6	8
None (Control)	0.8 ± 0.5	0.5 ± 0.4	1.6 ± 1.0	1.9 ± 0.7
1.25	0.9 ± 0.8	1.5 ± 0.3	3.0 ± 0.7	5.0 ± 0.5
2.5	0.8 ± 0.3	2.9 ± 1.3	4.4 ± 0.6	5.1 ± 1.3
5.0	2.1 ± 0.9	4.4 ± 0.5	5.8 ± 0.9	7.2 ± 0.4
10.0	1.9 ± 0.6	4.7 ± 0.2	6.5 ± 0.5	7.3 ± 0.6

Table 2: Effect of addition of increasing concentrations of exochelins on iron uptake by the avirulent ($H_{37}R_a$), virulent ($H_{37}R_v$) and the South Indian low virulent (SILV) strains of M. tuberculosis *at different weeks (means ± SD of 4 estimates).*

M. Tuberculosis strains	Exochelin concentration (µg mL^{-1})	Iron uptake (µg mL^{-1}) at the following weeks after inoculation			
		1	2	3	4
$H_{37}R_a$	None (control)	0.8 ± 0.3	1.4 ± 0.4	1.4 ± 0.7	1.9 ± 0.4
	1.25	0.8 ± 0.4	1.3 ± 0.5	2.5 ± 0.3	4.0 ± 0.5
	2.5	1.0 ± 0.4	1.3 ± 0.7	3.6 ± 0.8	4.5 ± 1.0
	5.0	0.7 ± 0.2	1.6 ± 0.8	5.1 ± 0.5	5.7 ± 0.3
	10.0	0.8 ± 0.4	2.0 ± 0.5	5.3 ± 0.3	6.4 ± 0.9
$H_{37}R_v$	None	0.9 ± 0.4	1.2 ± 0.5	1.4 ± 0.4	1.9 ± 0.6
	1.25	0.8 ± 0.3	1.4 ± 0.6	2.5 ± 0.5	3.0 ± 0.7
	2.5	0.9 ± 0.4	1.4 ± 0.2	2.4 ± 0.7	4.3 ± 0.6
	5.0	1.1 ± 0.4	2.6 ± 0.3	3.9 ± 0.8	6.6 ± 0.6
	10.0	1.7 ± 0.4	3.0 ± 0.4	4.5 ± 0.4	7.0 ± 0.6
SILV	None	0.7 ± 0.6	1.2 ± 0.6	1.6 ± 0.9	1.8 ± 0.7
	1.25	1.3 ± 0.4	2.0 ± 0.9	2.5 ± 0.6	3.7 ± 0.5
	2.5	1.0 ± 0.7	1.4 ± 1.0	2.8 ± 0.4	4.6 ± 0.4
	5.0	1.2 ± 0.7	1.5 ± 0.7	3.7 ± 0.6	5.2 ± 0.7
	10.0	1.4 ± 0.3	2.4 ± 0.4	3.9 ± 0.9	5.1 ± 0.3

up in quadruplicate and the estimations were undertaken after coding the flasks.

Residual iron concentrations in the media were determined using the SP 9700-atomic absorption spectrophotometer-flame system (Pye-Unicam Instruments, Cambridge, UK), at 2, 4, 6 and 8 days after inoculation with *M. smegmatis* and at the lst, 2nd, 3rd and 4th week after inoculation with the three *M. tuberculosis* strains.

The iron taken up by the bacilli was calculated as the difference between the initial concentration (15 μg mL^{-1}) and the residual iron concentration. The results were statistically analysed using Student's t-test (paired and unpaired).

Results: Addition of exochelins to media inoculated with *M. smegmatis* caused a significant increase in the uptake of iron only from the 4th day onwards ($p < 0.01$). The iron uptake by *M. smegmatis* was raised significantly ($p < 0.01$) with increasing concentrations of exochelins in the medium (Table 1). With the three *M. tuberculosis* strains, no significant differences were observed at the 1st and 2nd week; the uptake was significantly higher at the 3rd and 4th weeks ($p < 0.01$). The iron uptake by the three *M. tuberculosis* strains was also raised significantly ($p < 0.01$) with increasing concentrations of exochelins in the medium (Table 2).

The uptake of iron was higher with the saprophytic *M. smegmatis* than with any of three M. *tuberculosis* strains. Of the three *M. tuberculosis* strains, the uptake of iron was slightly higher with the virulent strain than with the avirulent or low virulent strains ($p = 0.02$).

Discussion: The uptake of iron from various siderophores into microbial cells is being increasingly well documented [8, 9]. The process of iron uptake in mycobacteria is distinct from the processes described in other bacteria [10], as two molecules with the ability to chelate iron are involved - the exochelins and the mycobactins. As salicylate is produced in considerable amounts under iron deficient conditions, it was thought to function as the external siderophore. However, salicylate has been found to be incapable of holding iron in solutions in the presence of phosphate ions [11]. It was later established that exochelins, found to be present in all species of mycobacteria studied, serve as the external siderophore [12].

Exochelin from *M. smegmatis* can solubilise iron from ferric phosphate and ferritin and mediate the uptake of iron by *M. smegmatis* [13]. Uptake of iron in *Azospirillum lipoferum* strain D-2 was stimulated rapidly by the addition of siderophores extracted from the organism to the medium [14]. The present uptake findings show an increase in the uptake of iron with increasing concentrations of exochelins in the medium by all four strains of mycobacteria, thus clearly establishing a role in iron-uptake for these compounds. Further, the uptake of iron was lower in the SILV strain than in the $H_{37}R_v$ strain. Whether the relatively low iron uptake by the SILV strain even in the presence of added exochelins is related to the expression of receptor proteins (iron-regulated envelope proteins) on the mycobacterial envelope is unknown, but their relevance to virulence needs to be investigated.

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