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Iron-regulated envelope proteins of mycobacteria grown in vitro and their occurrence in *Mycobacterium avium* and *Mycobacterium leprae* grown in vivo

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Summary. Several iron-regulated envelope proteins (IREPs), 11–180 kDa, have been detected in preparations of walls and membranes of *Mycobacterium smegmatis*, in an armadillo-derived mycobacterium (ADM) and in *M. avium*. The same sized proteins from *M. vaccae* appeared under both iron-deficient and iron-sufficient growth conditions. Two larger proteins, of 240 and 250 kDa, appeared in the membranes of *M. smegmatis* and *M. avium* only when grown iron-sufficiently but were constitutively present in both ADM and *M. vaccae*. The IREPs from *M. smegmatis* were not induced under zinc-deficient growth conditions. Three of the four IREPs (14, 21 and 29 kDa) recognized in *M. avium* grown in vitro were also recovered from membrane fractions of the same strain grown in mice. In addition, these membranes contained both the high-molecular-mass proteins associated with iron-sufficient growth conditions. Membranes of *M. leprae*, recovered from infected armadillos, showed the faint presence of a possible IREP at 29 kDa and wall preparations showed the presence of a 21-kDa protein. Membranes also contained the two larger proteins at 240 and 250 kDa. An explanation for the simultaneous occurrence of both low-iron-regulated and high-iron-regulated proteins is offered.

Key words: Iron deficiency - Mycobacteria - Leprosy bacillus - Envelope proteins - Acquisition (of iron) from animals

Introduction

The adaptation of many bacteria to an iron-restricted environment involves not only the synthesis of siderophores but also production of surface protein receptors and enzymes that are involved in uptake and release of iron from the siderophore. Specific membrane protein receptors for siderophores have been demonstrated in several micro-organisms under iron-restricted growth conditions (Braun et al. 1976; Brown et al. 1984; Chart et al. 1986; Cody and Gross 1987; Williams and Brown 1986). Mycobacteria, like other microorganisms, can adapt to growing under iron-restricted conditions and produce, under conditions of iron deficiency, a series of extracellular siderophores, termed exochelins, as well as a wholly intracellular lipid-soluble siderophore, mycobactin (Ratledge 1982; 1987). Concomitant with the production of exochelin and mycobactin, four iron-regulated envelope proteins (IREPs) have been shown (Hall et al. 1987) to be expressed by *Mycobacterium smegmatis* with apparent molecular sizes corresponding to 180 kDa (protein I), 84 kDa (protein II), 29 kDa (protein III) and 25 kDa (protein IV). Evidence has also been presented for of at least one of these proteins, protein III, being a receptor for ferri-exochelin as antibodies raised against this protein caused substantial ($\approx$70%) inhibition of iron uptake when pre-incubated with whole cells (Hall et al. 1987).

Pathogens, by their ability to cause infections, show that they have evolved ways of overcoming the natural restriction of iron imposed by their hosts (Griffiths 1983; 1985) and their ability to synthesize iron-regulated receptor proteins appears to be an important virulence factor (Brown et al. 1984; Griffiths et al. 1983). The expression of these iron-regulated proteins has been demonstrated in bacteria grown in vivo; for example, in those causing infections of the urinary tract (Lam et al. 1984; Shand et al. 1985), the peritoneal cavity (Griffiths et al. 1983) and in lung tissue of cystic fibrosis patients (Brown et al. 1984).

In this paper, we have examined the distribution of the IREPs among further mycobacterial species. These include *M. vaccae* which lacks mycobactin (Hall and Ratledge 1984), also the pathogen, *M. avium*, which has been grown in vivo and in vitro and finally in *M. leprae*, the leprosy bacillus, which cannot be grown in vitro and thus of necessity can only be recovered from animal tissues.

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Materials and methods

Organisms used. Organisms used were *M. smegmatis* NCIB 8548, *M. neoaurum* NCTC 10439, *M. avium* CR 1/69, *M. vaccae* R877R and an armadillo-derived mycobacterium (ADM) no. 8563 (see Portaels et al. 1986). *M. leprae* was isolated in the laboratory by Dr P. R. Wheeler from frozen (−80° C) armadillo livers following procedures outlined elsewhere (see for example Hall and Ratledge 1987).

Media and culture conditions. Mycobacteria were grown in a chemically defined glycerol/asparagine/salts liquid medium (100 ml in 250-ml conical flask), prepared as previously described for the removal of iron (Ratledge and Hall 1971), and shaken at 37° C at 200 rev./min for 3–7 days. For iron-deficient growth, iron was added at a concentration of 0.2 μg Fe/ml and for iron-sufficient growth, a supplement of 4.0 μg Fe/ml was added. For zinc-deficient growth, the usual supplement of Zn^{2+} (0.46 μg/ml) was excluded from the medium; iron was at 2 μg/ml.

M. avium grown in vivo. M. avium strain CR 1/69, recovered from the liver of a previously infected mouse, was injected intravenously into female C57 black mice 4–5 weeks old. The mice were sacrificed after 8–10 weeks and the mycobacteria recovered from the lungs, spleen and liver, following a procedure described for the recovery of *M. leprae* from infected animal tissue (Wheeler and Gregory 1980). (The *M. avium* used in this work was kindly supplied to us by Dr P. R. Wheeler.)

Preparations of membrane fraction and SDS/PAGE electrophoresis. The procedures followed were essentially those of Hall et al. (1987). Molecular mass standards (Pharmacia) included thyroglobulin (330 kDa), ferritin (two subunits 220 and 18.5 kDa), catalase (60 kDa), lactate dehydrogenase (36 kDa) and albumin (67 kDa).

Results

Iron-regulated envelope proteins elaborated by M. smegmatis

The appearance of the IREPs in the walls and membranes of *M. smegmatis* during the course of growth was followed from day 1 to day 7 (Fig. 1). Previous work (Hall et al. 1987) has shown four IREPs (180, 84, 29 and 25 kDa) were expressed in whole cell-envelope fractions of iron-limited *M. smegmatis*. In this present study, both the wall and membrane fractions of the envelope from similarly grown cells, when analysed by SDS/PAGE (Fig. 1a and b), revealed a fifth prominent protein of apparent molecular size 14 kDa. This was achieved by allowing electrophoresis to continue for 10–15 min after the bromophenol marker dye had reached the bottom edge of the glass plate. Additionally, the membranes but not the walls from cells grown iron-sufficiently showed two high-molecular-mass proteins (240 and 250 kDa) which were entirely absent in low-iron cells (Fig. 1b, lane H).

Some IREPs appeared simultaneously in the wall and membrane. For instance, the 29-kDa protein was seen after 2 days of growth in both wall and membrane fractions. Other IREPs appeared first in the membrane, then the wall: thus the 25-kDa and 14-kDa proteins were seen in the cell membrane from as early as day 1 of growth, while they were seen in the cell wall only

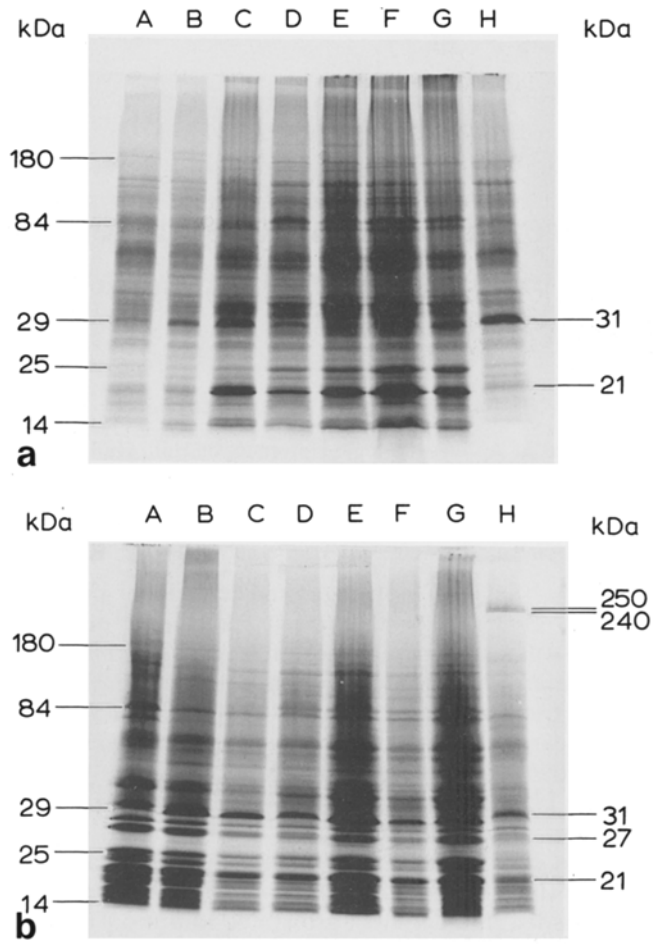

Fig. 1. SDS/PAGE analysis of **(a)** wall proteins and **(b)** membrane proteins (100 μg per lane) from iron-deficiently grown *M. smegmatis* cells harvested on days 1–7 (lanes A–G respectively). In each case lane H represents the equivalent protein fraction from iron-sufficiently grown cells harvested on day 7

after at least 3 days of growth. It is possible that these proteins are synthesised in the membrane and translocated to the cell wall. Some other proteins, e.g. the 27- and 21-kDa ones, were seen as prominent bands in the iron-deficient cell walls (Fig. 1a, lanes C–G) and, though inconspicuous in the corresponding walls of the iron-sufficiently grown cells (Fig. 1a, lane H), were seen to be more obviously present in the membrane preparations of the same cells (Fig. 1b, lane H) and thus have not been considered as true iron-regulated proteins.

All IREPs were repressed in cells grown iron-sufficiently (see Fig. 1a and b, lane H). The critical concentration for repression is approximately 0.5 μg iron/ml (Sritharan and Ratledge 1989).

To confirm that the IREPs of *M. smegmatis* arose solely in response to the iron deprivation in the medium and not as a result of a generalised stress induced by iron deficiency, the cells were also grown zinc deficiently but with a sufficiency of iron. Zinc deficiency can be easily induced in *M. smegmatis* (see Ratledge and Hall 1971) and was a better alternative than using magnesium limitation which may well have led to changes in cell wall structure. Examination of the mem-

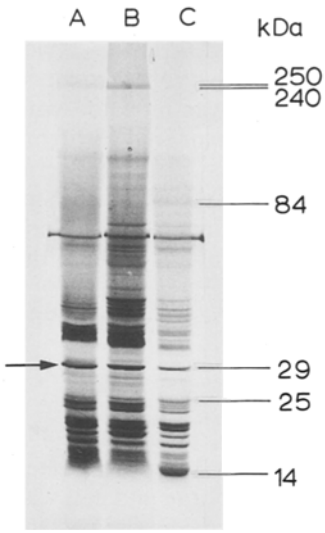

Fig. 2. SDS/PAGE analysis of cell membrane proteins (50 μg per lane) from *M. smegmatis*. Lanes: A, from cell grown iron-sufficiently; B, from cells grown zinc-deficiently; C, from cells grown iron-deficiently. The different IREPs are indicated by their molecular mass. The arrow indicates that the band in the zinc-deficient cells at about 29 kDa is in fact a protein of 31 kDa

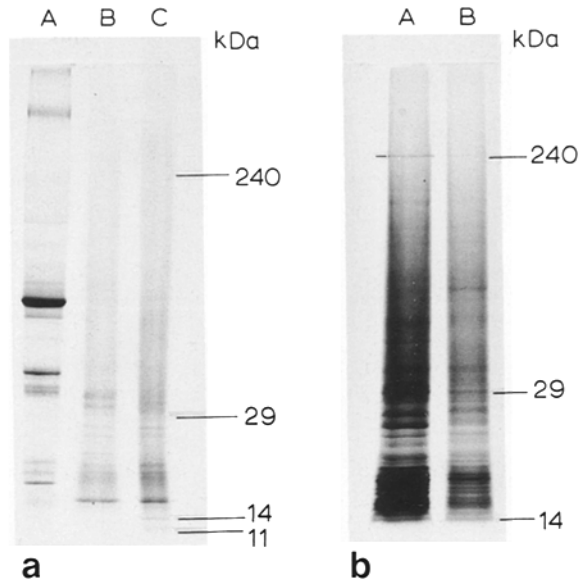

Fig. 3. Comparison of the cell membrane protein profiles of **(a)** armadillo-derived mycobacterium ADM 8563; and **(b)** *M. vaccae* R877 by SDS/PAGE. **(a)** Lane A, molecular mass markers as indicated (Pharmacia); lanes B and C = membrane proteins of ADM 8563 grown iron-sufficiently and iron-deficiently, respectively. **(b)** Lanes A and B = membrane proteins respectively from iron-sufficient and iron-deficient cells of *M. vaccae*, respectively

brane proteins from such cells, when analysed by SDS/PAGE (Fig. 2, lane B), failed to reveal proteins at 180, 84, 29 and 14 kDa though a protein of 25 kDa was still seen. The 240-kDa and 250-kDa proteins, associated with iron-sufficiently grown cells, were still present in the zinc-deficient cells. Some proteins that were seen in zinc-deficient cells had not been seen in the membranes of cells grown with a sufficiency of iron and zinc (i.e. Fig. 1a and b, lane H). No attempt was made to study these proteins further.

IREPs of other mycobacteria

The occurrence of IREPs in cell membrane fractions of several other mycobacteria was examined. *Mycobacterium* ADM 8563, originally recovered from the nine-banded armadillo (Portaels and Pattyn 1982), produces exochelin and mycobactin when cultivated in the laboratory (Hall and Ratledge 1985; 1987). It synthesized three IREPs upon iron-deprived growth (see Fig. 3a, compare lanes C and B). These were of 29, 14 and 11 kDa; the first two are similar in size to those proteins elaborated by iron-deficiently grown cells of *M. smegmatis*, *M. neoaurum* (Sritharan and Ratledge 1989) and *M. vaccae* (see below) but the third IREP, of 11 kDa, was possibly a new protein. All three proteins were repressed in iron-sufficiency grown cells (Fig. 3a, lane B).

The strain of *M. vaccae* which was used, R877R, does not synthesize the lipid-soluble siderophore mycobactin even when grown under iron limitation under a variety of different growth conditions (Hall and Ratledge 1984; 1986). The membrane protein profile of this strain did not reveal any additional proteins which were synthesized exclusively by iron-deficient cells (Fig. 3b, compare lanes B and A). Proteins of 29 kDa and 14 kDa, characteristic of iron-deficiency in the other mycobacteria, appeared to be expressed in both iron-sufficient and iron-deficient cells. The 240-kDa protein, characteristic of iron-replete growth in the other mycobacteria, was also found to be expressed in both high and low iron cells though the band was

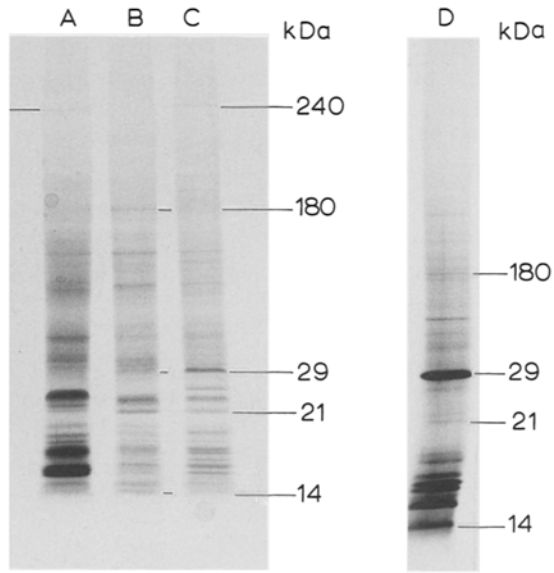

Fig. 4. Comparison of the cell envelope proteins by SDS/PAGE of *M. avium* grown in vivo with cells grown in vitro. Lanes A and B represent cells grown in vitro iron-sufficiently and iron-deficiently, respectively. Lanes C and D represent the cell membrane and wall fractions, respectively, of *M. avium* grown in vivo (recovered from infected mouse tissue)

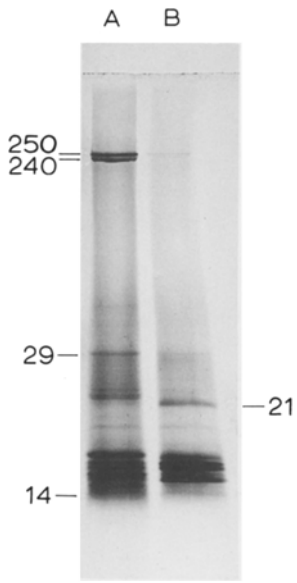

Fig. 5. SDS/PAGE of the proteins of the cell membrane (lane A) and cell wall (lane B) from *M. leprae* recovered from livers of infected armadillos

fainter (and is just discernable in the illustration) in the iron-deficiently grown cells.

IREP elaborated by M. avium *grown in in vitro and in vivo*

M. avium, though not considered virulent in man, is the causative organism of tuberculosis in birds. Mice can also be infected by it. Membrane proteins, isolated from *M. avium* grown in vitro under iron-deficient conditions, when analysed by SDS/PAGE, showed the presence of four IREPs with molecular masses of 180, 29 (faintly), 21 and 14 kDa (Fig. 4, compare lanes A and B). When the proteins were analysed from *M. avium* recovered from infected mice, three of these proteins, the 29-kDa, 21-kDa and 14-kDa ones, were seen in the membrane fraction (Fig. 4, lane C). However, the other IREP, the 180-kDa IREP was not in evidence. Furthermore, the large 240-kDa protein, characteristic of iron-sufficient growth in vitro (Fig. 4, lane A), was also seen in the membranes from the cells grown in vivo (Fig. 4, lane C). This isolation was repeated with the same result using mycobacteria recovered just from the liver of several mice. Preparations of wall proteins from *M. avium* grown in vivo showed the two IREPs of 14 kDa and 29 kDa to be very prominent (Fig. 4, lane D) with the 180-kDa and 21-kDa proteins to be quite faint.

IREPs in M. leprae *grown in armadillos*

Mycobacterium leprae, the causative organism of human leprosy, was recovered from the livers of infected armadillos. The proteins of cell membrane and cell wall fractions, when examined by SDS/PAGE, showed the strong presence of a 21-kDa protein in the cell wall and a 29-kDa protein in the membrane together with a fainter protein at 14 kDa (Fig. 5). Very strikingly, the proteins at 240 and 250 kDa, associated with iron-sufficient growth of the other mycobacteria, were clearly in evidence in the cell membrane fraction but were only faintly seen in the wall preparation.

Discussion

The distribution of the IREPs among the mycobacteria studied both here and previously (Sritharan and Ratledge 1989) is summarized in Table 1. Proteins of similar size (180, 29 and 14 kDa) are expressed in most species examined. However, the equivalence and functional significances of these proteins have yet to be established. Some IREPs are, however, synthesized only by some mycobacteria: for example, the 11-kDa protein was seen only in the ADM strain, the 84-kDa protein in *M. smegmatis* and, previously, the 120-kDa protein only in *M. neoaurum* (Sritharan and Ratledge 1989).

The two high-molecular-mass proteins (of 240 and 250 kDa) associated with iron-sufficient growth in vitro were expressed in all species studied including *M. le-*

Table 1. Summary of the occurrence of the different IREPs analysed by SDS/PAGE in mycobacteria

Organism	Presence of IREPs of									
	240 kDa	250 kDa	180 kDa	120 kDa	84 kDa	29 kDa	25 kDa	21 kDa	14 kDa	11 kDa
M. smegmatis	*	*	+	–	+	+	+	–	+	–
M. neoaurum[a]	*	*	?	*	–	+	–	+	+	–
M. vaccae	Δ	Δ	–	–	–	Δ	Δ	–	Δ	–
ADM 8563	?	?	–	–	–	+	–	–	+	+
M. avium (in vitro)	*	*	+	–	–	+	–	+	+	–
M. avium (in vivo)	/	/	/	–	–	/	–	/	/	–
M. leprae (in vivo)	/	/	–	–	–	/	–	/	/	–

+ and – denote the presence or absence of the IREP in iron-deficiently grown cells; * represents the expression of the respective protein only in iron-sufficient cells; Δ represents the expression of the respective protein in both iron-sufficient and iron deficient cells; ? very faint band, uncertain presence; / indicates presence in vivo where iron status is uncertain (see Discussion for possible explanation)

[a] Data from Sritharan and Ratledge 1989

prae. However, in *M. vaccae* and ADM 8563, they appeared to be constitutively expressed irrespective of the degree of available iron. (In the illustration of the IREPs of the latter organism, Fig. 3a, these protein bands are very faint but were more obvious in other runs.) The function of these proteins is not clear though Klebba et al. (1982) suggested that a protein of 90 kDa found in iron-sufficiently grown *Escherichia coli,* but not in iron-deficiently grown cells, could have a ferritin-like role in iron storage. It would thus be of interest to determine if these proteins in mycobacteria fulfil a similar role.

M. vaccae was anomolous for also not repressing the biosynthesis of the small IREPs (29, 25 and 14 kDa) which had been found in the other mycobacteria (Table 1). ADM 8563 however did repress formation of all its IREPs. Why *M. vaccae* should behave in this way in unclear though, of all the mycobacteria examined here, it alone does not produce mycobactin (Hall and Ratledge 1984; 1986). If some or all of the IREPs had been connected with mycobactin biosynthesis, these proteins may then have been expected to be absent in *M. vaccae* under any growth condition. The appearance of the proteins in *M. vaccae* would therefore suggest that they are unconnected with mycobactin biosynthesis or function though their constitutive appearance remains to be explained. With respect to other aspects of iron metabolism, *M. vaccae* does not appear to be in any way exceptional (Messenger et al. 1986).

M. avium recovered from infected mice possessed some, but not all, of the IREPs which had been found to be expressed by cells grown in vivo. With *M. leprae*, because it cannot be cultivated in the laboratory, no such comparison can be made but there was clear evidence for a 21-kDa protein occurring in the cell wall and a 29-kDa protein occurring in the membrane fraction. Whether these proteins are IREPs has yet to be determined but we have already established that *M. leprae* can take up Fe(III) when coordinated to the exochelins from ADM 8563 or from *M. neoaurum* (Hall and Ratledge 1987) and presumably therefore must use appropriate exochelin-receptor proteins (the IREPs?) for this. Whether the 29-kDa protein corresponds to the recently described 28-kDa proteins of *M. leprae* considered to be a major target for antibodies from patients with lepromatous leprosy (Cherayil and Young 1988) is entirely speculative at this stage but clearly this possibility should be examined.

Although this evidence may suggest that both *M. avium* and *M. leprae* grow in vivo in an iron-deficient environment, both pathogens clearly expressed the high-molecular-mass proteins of 240 and 250 kDa associated with iron-sufficient growth when recovered from infected tissues. An explanation for this puzzling simultaneous occurrence of low-iron and high-iron-regulated proteins can, though, be offered.

Perhaps mycobacteria when in vivo are initially in an iron-deprived environment but as they commence synthesis of exochelins, IREPs and mycobactins (probably coordinately, see Sritharan and Ratledge 1989) iron becomes available and thus then begins to repress the biosynthesis of the very molecules responsible for its assimilation. It is thus not difficult to imagine that mycobacteria, within an animal tissue, are on the cusp of iron deprivation/iron sufficiency and balance the synthesis of iron assimilatory components with the increased availability of iron which then ensues. The synthesis of the iron uptake components need never be as extensive as is seen in the artificially contrived iron-deficient growth conditions of a laboratory culture where iron is permanently withheld from the cells. Such a situation does not occur in vivo. The iron uptake components are synthesized but their very synthesis leads to increased iron availability and assimilation. Hence mycobacteria recovered from infected animal tissues show the presence of both the low-iron envelope proteins (the IREPs) as well as the high-iron proteins at 240 and 250 kDa, indicating that they exist at the stage between iron deprivation and assimilation.

Although we have so far only examined a few mycobacteria and have succeeded in implicating only one IREP (namely the 29-kDa protein from *M. smegmatis*) in exochelin-mediated iron uptake (Hall et al. 1987), it is nevertheless clear that specific envelope proteins are being synthesized by mycobacteria in response to iron deficiency. These results have implications for the possible preparation and use of specific antibodies, perhaps linked to bacteriocidal agents, either for early detection of mycobacterial diseases or in their chemotherapy. The results also imply that vaccines prepared from mycobacteria should use iron-deficiently grown cells rather than cells grown in an undefined manner in order to have the key proteins associated with iron acquisition properly expressed. Acquisition of iron is a key determination of bacterial virulence (Griffiths 1983; 1985) and our results suggest that mycobacteria are probably no different in this respect than other bacterial pathogens.

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