A new extracellular protein of *Pseudomonas aeruginosa* PA103 regulated by *regA*

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The expression of exotoxin A (ExoA) from *Pseudomonas aeruginosa* is influenced by iron and is under the control of the regulatory gene *regA*. To test whether *regA* plays a role in the expression of other iron-regulated proteins a *RegA*[−] mutant was constructed by insertional mutagenesis. The polypeptide pattern of this mutant (PA103R) was compared with the parental strain (PA103) and a *trans*-complemented strain PA103R(pREX18) after growth of the strains in conditions containing low or high concentrations of iron. An iron-regulated 42 kDa protein (RRP) was identified and purified from the culture supernatant of PA103 and PA103R(pREX18) which was missing in PA103R. Database analysis of the N-terminal sequence of this *regA*-regulated protein (RRP) revealed no similarity to other proteins. Preliminary investigations into the function of RRP revealed that it has no proteolytic or cytotoxic activity. Using two-dimensional electrophoretic analysis of whole cells, a technique which allowed separation of over 600 polypeptides, we were unable to identify any other iron-regulated protein whose expression was regulated by *regA*.

Keywords: *Pseudomonas aeruginosa*, *regA*, gene regulation, iron-regulated proteins

INTRODUCTION

Bacteria respond to environmental changes with co-ordinate gene expression (Miller *et al.*, 1989) using different signals and gene regulators. Also, the synthesis of virulence factors in *Pseudomonas aeruginosa* is influenced by various growth parameters. For example, production of exotoxin A (ExoA), a major virulence factor, is repressed under high iron conditions (Bjorn *et al.*, 1978) and depends on the regulatory genes *regA* and *regB*, which are organized in an operon (Hedstrom *et al.*, 1986; for review, see Wick *et al.*, 1990). *regA* regulates ExoA *in trans* at the level of transcription (Frank & Iglewski, 1988; Frank *et al.*, 1989; Wick *et al.*, 1990) by an unknown mechanism. There is no evidence for binding of the *regA* gene product RegA to the upstream region of the ExoA gene *toxA* (Hamood & Iglewski, 1990). Transcript accumulation of *regA* itself is enhanced in low iron medium and precedes *toxA* transcription and translation (Frank & Iglewski, 1988; Hindahl *et al.*, 1988). The transcription of *regA* involves two promoters, P1 and P2. Only the P2 promoter is strongly iron-regulated (Storey *et al.*, 1990, 1991).

Iron not only regulates ExoA synthesis but also affects the expression of other secreted proteins (Bjorn *et al.*, 1979), of proteins from the outer membrane and of secondary metabolites. These phenomena may be linked in *P. aeruginosa* to a common regulatory mechanism such as the regulation of iron-regulated proteins by Fur in *Escherichia coli* (Bagg & Neilands, 1987; Hantke, 1984). Recently a protein, analogous to Fur, was detected in *P. aeruginosa* and the gene cloned and sequenced (Prince *et al.*, 1991, 1993). Mutants producing altered Fur proteins were selected and these mutants constitutively produced siderophores and ExoA under high iron conditions that usually repressed their production (Prince *et al.*, 1993). Introduction of the *fur* gene from *E. coli* in *P. aeruginosa* inhibited the transcription of *regA* from the P1 promoter as well as ExoA synthesis (Prince *et al.*, 1991). Interestingly, transcription of the iron-regulated P2 promoter of *regA* was not regulated by *fur*.

regA as well as *fur* are candidate genes as global regulators for iron-regulated proteins in *P. aeruginosa*. At present, besides ExoA no other protein has been shown to be regulated by *regA*. To investigate the role of *regA* in this context we constructed a *RegA*-negative mutant PA103R and compared the protein expression of this strain with

Abbreviations: ExoA, exotoxin A; RRP, *regA*-regulated protein.

Table 1. Bacteria and plasmids used in this study

Organism	Characteristics	Reference
<i>E. coli</i>		
S17-1	MM294 with RP4 2-Tc::Mu-Km::Tn7 integrated into the chromosome	Simon <i>et al.</i> (1983)
HB101	<i>recA13 hsdS20</i> (r _B ⁻ m _B ⁻)	Sambrook <i>et al.</i> (1989)
<i>P. aeruginosa</i>		
PA103	Prototroph	Liu (1966)
PA103R	PA103 with pBRMOB-RegA integrated in <i>regA</i>	This study
PA103R(pREX18)	PA103-R complemented with pREX18	This study
Plasmids		
pSUP201-1	<i>bla cml mob</i>	Simon <i>et al.</i> (1983)
pBRMOB	pBR322 ligated with 1·8 kb <i>Bam</i> HI <i>mob</i> fragment from pSUP201-1	This study
pBRMOB-RegA	pBRMOB ligated with 366 bp <i>Sal</i> I- <i>Sal</i> I fragment from pUCEE 8·5	This study
pREX18	<i>bla</i> Sm ^R Su ^R <i>regA</i>	Vasil <i>et al.</i> (1989)
pRK2013	Helper plasmid, RK2 transfer gene with ColE1 replicon	Figurski & Helinski (1979)

the parental strain PA103 and the *regA*-complemented strain PA103R(pREX18).

METHODS

Strains, plasmids and glassware. Strains and plasmids used in this study are listed in Table 1. For all iron-restricted growth experiments the glassware was rinsed in 1 mM EDTA overnight followed by one rinse with 0·1 M HCl and six with distilled H₂O.

Construction of RegA mutant of PA103. For insertional mutagenesis the mobilization vector pBRMOB-RegA was constructed. A 1·9 kb *Bam*HI fragment carrying the recognition site of mobilization from pSUP201-1 (Simon *et al.*, 1983) was isolated and cloned into the *Bam*HI site of pBR322 resulting in pBRMOB. The internal 366 bp *Sal*I-*Sal*I fragment (coding amino acids 52–174) of the *regA* gene was subsequently ligated with the *Sal*I site of pBRMOB and transformed into *E. coli* strain S17-1 which carries the *tra* genes of the broad-host-range plasmid RP4 integrated in the chromosome (Simon *et al.*, 1983). pBRMOB-RegA was then transferred from *E. coli* to *P. aeruginosa* in filter matings (Vasil *et al.*, 1989). Transconjugants were selected on cetrinide agar plates (selective *P. aeruginosa* agar, Difco) containing carbenicillin (500 µg ml⁻¹). The plasmid pBRMOB-RegA is only replicated in *P. aeruginosa* when integrated into the chromosome by homologous recombination with *regA*. This was confirmed by Southern blot analysis of purified chromosomal DNA digested with *Xho*I and hybridized with the internal 366 bp *Sal*I fragment from the *regA* coding region. DNA methods were performed according to Sambrook *et al.* (1989), or the instruction of the manufacturer of the detection kit (Boehringer Mannheim).

Complementation of the *regA* mutation in PA103R. For complementation of the chromosomal *regA* mutation *in trans* the *regA* gene was transferred into PA103R by triparental mating between the donor strain *E. coli* 294(pREX-18) (Vasil *et al.*, 1989), the *E. coli* helper strain HB101(pRK2013) and the

recipient *P. aeruginosa* PA103R. The plasmid pREX-18 contains the coding region of *regA* integrated into pUC18 and fused with the broad-host-range vector RSF1010. Mating was carried out as described by Vasil *et al.* (1989). Colonies were picked and analysed by radioimmunoassay (Döring *et al.*, 1985) and immunoblotting for ExoA synthesis. The *trans*-complemented strain was designated PA103R(pREX18).

Media and growth conditions. *E. coli* was grown in Luria-Bertani medium (Sambrook *et al.* 1989) supplemented with the appropriate antibiotics (100 µg ampicillin ml⁻¹). *P. aeruginosa* was grown in chelated trypticase soy broth (cTSB) (Kadurugamuwa *et al.*, 1987). Double-strength trypticase soy broth (Oxoid) was prepared in 0·5 l batches and passed twice through a Chelex-100 column (Bio-Rad) (200 ml bed volume) and finally adjusted to 1 l with the water eluate of the column. To the medium 1% (v/v) glycerol, 8·5 µM ZnSO₄, 1·3 mM MgSO₄ and 140 µM CaCl₂ was added and the pH was adjusted to 7·4 before autoclaving. The iron concentration in the medium was 125 µg l⁻¹ as determined by atomic absorption. For iron-sufficient growth conditions, 45 µM FeSO₄ was added from a freshly prepared sterile filtered stock solution just before inoculation of the bacteria. Bacteria were inoculated in 25 ml medium into 100 flasks at an initial OD₄₈₀ of 0·01 from a 10 h culture in the same medium. The mutant PA103R was precultured with 1000 µg carbenicillin ml⁻¹, strain PA103R (pREX18) with carbenicillin and streptomycin (1000 µg ml⁻¹ each). Growth was followed by measuring the OD₄₈₀ at 1 h intervals. As a control for purity and genetic stability all strains were plated on TSB agar with and without carbenicillin or streptomycin (1000 µg ml⁻¹) after growth.

Cells were harvested by centrifugation (4300 g, 10 min). The few remaining cells were removed by a second centrifugation of the culture supernatant fluid (15000 g, 30 min). Cells were suspended in 0·85 M NaCl supplemented with 15% glycerol (1 ml per 10⁹ cells), and frozen in aliquots at -80 °C until use. Culture supernatant (10 ml) was dialysed against 1 l distilled H₂O overnight and dried in a vacuum concentrator or stored in

aliquots until use. For electrophoresis the concentrate was resolved in 1 ml of sample buffer (Laemmli, 1970).

SDS-PAGE and immunoblotting. Proteins were separated using polyacrylamide gels containing SDS (Laemmli, 1970) with 3% (w/v) crosslinking monomer concentration (C) and 10% total monomer concentration (T). The gels were stained with colloidal Coomassie blue G (Neuhoff *et al.*, 1985) or with silver (Blum *et al.*, 1987). For immunoblotting, proteins were transferred to nitrocellulose (Schleicher & Schuell) using a semidry blotting system (Millipore). Two different rabbit antibody preparations against ExoA served as primary antibody (Döring *et al.*, 1985; Bourdenet *et al.*, 1990). Immunoblots were developed with peroxidase-conjugated secondary antibodies (Dako-Diagnostika) in combination with chemoluminescence according to the manufacturer (Amersham Buchler).

Two-dimensional electrophoresis. This was performed on a horizontal system (Multiphore, Pharmacia) with an immobilized pH gradient in the first dimension and SDS-PAGE in the second dimension (Görg *et al.*, 1988). The isoelectric focusing (IEF) gel (pH gradient 4–9) was prepared and dried according to the instructions of the manufacturer (Pharmacia) and stored at -20°C until use. Strips (5 mm) were cut and reswollen in 8 M urea, 0.5% NP-40, 13 mM DTT and 0.2% Biolyte 3/10. Whole cells (10^9) were added to 1 ml solubilizing mix (9 M urea, 2% NP-40, 65 mM DTT and 0.8% Biolyte 3/10) and 50 μl was applied to the IEF gel strips with the dry strip kit (Pharmacia). After electrophoresis (30 min 150 V, 60 min 300 V, 60 min 1500 V, 30 min 2500 V, 3 h 3000 V, 2 h 3500 V; 0.05 mA per strip) the strips were stored at -70°C until use for SDS-PAGE. The SDS-polyacrylamide gels were prepared with 4% C, 12–15% T according to Laemmli (1970). Gels were stained with silver by the method of Blum *et al.* (1987). Experiments were repeated at least three times. Analysis of the polypeptide pattern was performed visually by two individuals.

Purification of the 42 kDa protein from the culture supernatant fluid of PA103. PA103, PA103R and PA103R(pREX18) were grown in cTSB and 30 ml of culture supernatant fluids were diluted 1:4 with ice-cold distilled H_2O . DEAE-Sepharcel (Pharmacia) (20 g) was swollen with 0.02 M Tris/HCl pH 7.8, 0.01 M NaCl and added to each dilution and rotated for 2 h at 4°C . The material was then packed in a column and washed with 0.02 M Tris/HCl, 0.01 M NaCl, pH 7.8. The pass-through fraction was pooled, and the column eluted with increasing NaCl concentrations (0.05 M, 0.1 M, 0.15 M, 0.2 M, 0.25 M, 1 M, each in 0.02 M Tris/HCl, pH 7.8). All fractions were pooled, concentrated ($10\times$) by ultrafiltration (10 kDa-Unit, Amicon) and subjected to SDS-PAGE. RRP was detected only in the pass-through fractions of PA103 and PA103R(pREX18). The pass-through fraction from the DEAE-Sepharose column was dialysed against 0.02 M sodium phosphate buffer, pH 7.8, and subjected to Hiload S-Sepharose column (Pharmacia) equilibrated with 0.01 M NaCl, 0.02 M sodium phosphate buffer pH 7.8. Proteins were eluted with increasing NaCl concentrations (0.1 M, 0.25 M, 0.5 M, 1 M, in 0.02 M phosphate buffer pH 7.8). Fractions were pooled, concentrated and analysed by SDS-PAGE. In the 0.25 M fraction a 42 kDa protein (RRP) was detected in PA103 and PA103R(pREX18) but not in PA103R.

In a second purification procedure, 30 ml of the culture supernatant fluids were dialysed against 0.01 M NaCl, 0.02 M phosphate buffer pH 7.8, and directly applied to the equilibrated S-Sepharose column. Again, RRP was present in the 0.25 M NaCl fraction from PA103R(pREX18) but not from PA103. The RRP fraction was separated by SDS-PAGE (10% T, 2.6% C) and transferred to Immobilon P membrane (Millipore) by semidry-blotting. The membrane was stained with amido black

(Serva), the 42 kDa protein band cut out and the N-terminal amino acid sequence determined by automated Edman degradation (model 470A gas-phase sequencer equipped with a 120 PTH amino acid HPLC analyser).

Functional assays for RRP. For preliminary characterization of RRP in supernatants of PA103, the azocasein assay (Prestigde *et al.*, 1971), radioimmunoassays for detection of alkaline proteinase (Döring *et al.*, 1982) and elastase (Obernesser & Döring, 1982), and a cytotoxic assay (Döring & Müller, 1989) were carried out. Dialysed PA103 and PA103R supernatants were used for the investigation of proteolytic activity in the azocasein assay. For the investigation of cytotoxicity, excess specific antibodies against ExoA were added to the supernatants and preincubated for 1 h at 37°C . In order to investigate an enhancing effect of RRP on ExoA, purified ExoA equivalent to the concentration of ExoA in PA103 was added to the supernatant of PA103R and cytotoxicity assessed in the CHO cell assay.

RESULTS

Construction of the RegA mutant PA103R by insertional mutagenesis of *Pseudomonas aeruginosa* PA103

The chromosomal gene *regA* from *P. aeruginosa* PA103 was interrupted by homologous recombination. The integration of plasmid pBRMOB-RegA into *regA* in the *P. aeruginosa* transconjugant PA103R was confirmed by filter hybridization of chromosomal DNA digested with *Xba*I (data not shown). Since there is no *Xba*I restriction site within the coding region of *regA*, integration of the plasmid leads to an increase in the size of the *regA*-specific fragment by 6.5 kb (the size of the plasmid). Evidence that pBRMOB-RegA had only integrated in *regA* was shown by the presence of a single hybridizing band. The mutation was complemented by introduction of the plasmid pREX18, carrying *regA*, and resulted in strain PA103R(pREX18).

Effect of *regA* on growth of *Pseudomonas aeruginosa*

No differences in doubling time or cell density in the stationary phase were seen between PA103, PA103R and PA103R(pREX18), indicating that *regA* has no influence on bacterial growth. When the iron-depleted medium (cTSB) was supplemented with iron, the cultures reached a higher cell density at stationary phase, indicating that cTSB was iron-limited (Table 2).

Effect of iron and *regA* on ExoA synthesis in *Pseudomonas aeruginosa*

ExoA concentration was determined by radioimmunoassay in the culture supernatant fluids of strains PA103, PA103R and PA103R(pREX18) after growth in cTSB with and without added iron. As expected, ExoA production decreased significantly when iron was added to the iron-depleted medium. The interruption of *regA* in PA103R resulted in an ExoA repression to 1.6% of the wild-type level. Addition of iron to the medium led to a

Table 2. Cell density and ExoA synthesis of *P. aeruginosa* PA103, PA103R and PA103R(pREX18)

All values shown represent the mean and SD of at least three experiments with bacteria grown on different days.

Strain	OD ₄₈₀ (stationary phase)*		ExoA† (µg ml ⁻¹)	
	Low iron	High iron	Low iron	High iron
PA103	8.18 ± 0.53	18.1 ± 0.8	21 ± 2.9	3.7 ± 0.91
PA103R	8.03 ± 0.86	18.8 ± 1.2	0.4 ± 0.1	0.2 ± 0.04
PA103R(pREX18)	7.86 ± 0.57	18.2 ± 1.4	22 ± 4.3	3.8 ± 0.98

* Cells were grown in cTSB to stationary phase (12 h) with no added iron (low iron) or 45 µM FeSO₄ (high iron). OD₄₈₀ = 1 corresponds to 1.5 × 10⁹ c.f.u. ml⁻¹.

† ExoA in the culture supernatant fluids was determined by radioimmunoassay after growth of the cells to stationary phase.

Fig. 1. Two-dimensional electrophoresis of whole cell proteins from *P. aeruginosa* PA103R (a) and PA103R(pREX18) (b) after growth in cTSB. The isoelectric focusing gel in the first dimension contained a pH gradient of 4–9.

slight further reduction in ExoA synthesis in PA103R, but this inhibition was less than the 83% decrease of ExoA caused by the addition of iron to PA103. The complementation of the *regA* mutation in PA103R(pREX18) restored iron-regulated ExoA synthesis to a degree comparable to PA103 (Table 2).

Effect of *regA* on the protein patterns of whole *Pseudomonas aeruginosa* cells

Extracts from whole cells were subjected to SDS-PAGE and the protein patterns of PA103 and PA103R were compared. Addition of iron in cTSB resulted in differences

in the protein pattern compared to the iron-depleted medium. However, no difference between PA103 and PA103R was seen under either growth condition. Two-dimensional electrophoresis was used to get a better resolution of the complex protein pattern of whole cells. Approximately 600 polypeptides could be visualized by this technique. Typical gels of proteins from PA103R and PA103R(pREX18) are shown in Fig. 1. The analysis of three independent sets of experiments did not reveal significant differences between the strains. Although spot density may differ from gel to gel due to irregular staining, spots missing in PA103R, but present in PA103 or PA103R(pREX18) and *vice versa* were not detectable.

Fig. 2. SDS-PAGE (Coomassie blue stain) of culture supernatant proteins from *P. aeruginosa* strains grown in cTSB without iron (lanes 1, 2 and 3) or with 45 µM FeSO₄ (lanes 4 and 5). Lanes 1 and 4, PA103; lanes 2 and 5, PA103R; lane 3, PA103R(pREX18).

Fig. 3. Purification of the *regA*-regulated protein (RRP) analysed by SDS-PAGE (silver staining). Lane 1, molecular mass markers (Pharmacia: 94, 67, 43, 30, 20 kDa); lanes 2, 3 and 4, pass-through fraction after DEAE-Sepharose chromatography; lanes 5 and 6, eluate from HiLoad S-Sepharose column with 0.25 M NaCl, 0.02 M phosphate buffer, pH 7.8; lanes 2 and 6, PA103; lanes 3 and 5, PA103R; lane 4, PA103R(pREX18).

Thus, we did not find cell-associated proteins which are regulated by *regA*. In contrast, there were 69 spots (data not shown) detectable in PA103 as well as in PA103R, which were dependent on the iron concentration in the medium.

Effect of *regA* on proteins of culture supernatant fluids

Analysis of the secreted proteins in the culture supernatant fluids showed that the total amount of secreted protein was lower under high iron conditions taking into consideration that the final cell density is higher under

these conditions. *regA* had no effect on alkaline proteinase, since this proteinase was present in equal amounts in the supernatants of PA103 and PA103R. Elastase was not detectable in either supernatant. However, *regA* mutagenesis in *P. aeruginosa* PAO1 revealed no difference in elastase concentrations between PAO1 and its RegA-deficient mutant (data not shown).

Interestingly, the *regA* mutant PA103R was deficient in one prominent negatively iron-regulated protein compared to PA103 and PA103R(pREX18) (Fig. 2). Since this 42 kDa protein did not react with ExoA-specific polyclonal antibodies in immunoblotting it was regarded as a second *regA*-regulated protein besides ExoA and was named RRP. RRP had no proteolytic activity as shown by the azocasein assay. Experiments in the absence and presence of specific antibodies against ExoA revealed that RRP had no cytotoxic activity (data not shown). Furthermore, addition of purified ExoA to culture supernatants of PA103R in concentrations found in PA103 revealed no significant difference in cytotoxic activity compared to PA103 supernatant. Thus, we conclude that RRP has no ExoA enhancing activity.

Purification and N-terminal sequence of RRP

Since RRP could not be identified by comparison with secreted proteins described in the literature, the protein was purified and its N-terminal sequence determined. For purification, culture supernatant fluids of PA103, PA103R and PA103R(pREX18) were subjected to ion-exchange chromatography. All steps were done in parallel and each purification step was controlled by comparing protein fractions of PA103, PA103R and PA103R(pREX18) by SDS-PAGE (Fig. 3). The protein passed through the anion exchanger (DEAE-Sepharose) at pH 7.8, but was bound to the cation exchanger (S-Sepharose) at pH 7.8, indicating a relatively basic pI of the protein. The protein could be eluted from the S-Sepharose column with 0.25 M NaCl. The same procedure performed with the culture supernatant fluid of PA103R revealed no equivalent protein. The N-terminal sequence determined after blotting the protein onto a polyvinylidene difluoride membrane was XXX-Glu-Thr-Ala-Gly-Lys-Leu-Pro-XXX-Lys-Pro-Ala-Leu-Gly-Pro-Glu. Database analysis revealed no obvious homology to any known protein sequence (SwissProt release 23, NBRF-PIR release 33, EMBL 32, GenBank 13).

DISCUSSION

As a tool to study the role of the *regA* gene in *P. aeruginosa*, insertional mutagenesis was used to interrupt the structural gene of *regA*. The interruption of the regulatory gene *regA* caused repression of ExoA synthesis to 1.6% of the wild-type level seen in strain PA103. This is comparable with previous results obtained with the putative *regA* mutant PA103-29 (Ohman *et al.*, 1980), which produced < 1% ExoA compared to PA103. The remaining ExoA synthesis in the mutant PA103R was slightly iron-regulated. This may be due to regulation by

a Fur analogue since this protein has been detected in *P. aeruginosa* and may bind to the consensus sequence of the Fur binding site at the *toxA* locus (Prince *et al.*, 1991).

Besides ExoA, another *regA*-regulated protein (RRP) was detected in the culture supernatant fluid of PA103. Evidence is provided by the following results. First, the protein was not detectable in PA103R, but was present in high concentrations in the culture supernatant of the parental strain PA103 as well as in the *trans* complemented strain PA103R(pREX18). Second, the synthesis of this protein was strongly negatively iron-regulated and RRP was not detectable in *P. aeruginosa* PAO1 (data not shown), which also produces less ExoA.

RRP is not a fragment of ExoA since polyclonal antibodies against ExoA did not cross-react with this protein. Furthermore the N-terminal sequence of RRP showed no homology with the ExoA sequence. The nature of this protein remains unclear since database analysis gave no indication of sequence homology to other proteins. A preliminary characterization of RRP revealed that this protein had no proteolytic, or cytotoxic activity. Furthermore, it did not enhance ExoA activity on CHO cells.

A 43 kDa protein was described by Ohman *et al.* (1980) in the supernatant of PA103 which was undetectable in a *regA* mutant PA103-29. This protein showed ADP-ribosyltransferase activity which could be inhibited with antiserum against ExoA and thus was thought to be an Exo A fragment. Most likely, Ohman *et al.* (1980) co-purified RRP together with this ExoA fragment by the gel elution technique. We were also able to detect such an ExoA fragment in the supernatant of PA103. This fragment was detectable only by sensitive immunoblotting with antibodies against ExoA and had a slightly higher molecular mass than RRP.

No effects of *regA* on the expression of other iron-regulated proteins were seen when using one- and two-dimensional gel electrophoresis. Thus, *regA* does not seem to be a global regulator in *P. aeruginosa* and therefore the effects of iron on cell metabolism are likely to be mediated by other regulatory mechanisms such as the *fur* gene homologue (Prince *et al.*, 1993) or by other, still unknown, genes. The questions whether *regA* regulates RRP transcription, translation and/or processing have not been addressed in the present study. The results from two-dimensional electrophoresis suggest that RRP does not accumulate in the cytosol of PA103R. Further experiments using specific antibodies to RRP are needed to clarify this and other issues concerning RRP.

ACKNOWLEDGEMENTS

This study was supported by a grant (Do 249/5) from the Deutsche Forschungsgemeinschaft to R.L. and C.W. and a grant from NIH AI15940 to M.L.V. The authors would like to thank the Chemical laboratory of the Hygiene Institute, Tübingen, for determination of iron by atom absorption, D. Roecklin, Transgene Spa, Strasbourg, France, for technical assistance, Dr A. Görg, Technische Universität München, Freising-Weihenstephan, Germany, for the very helpful advice

on two-dimensional electrophoresis and Dr M. Guinand, University of Lyon, France, for providing rabbit polyclonal antibodies against exotoxin A.

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Received 16 September 1993; revised 11 January 1994; accepted 25 January 1994.