



**Universiteit
Leiden**
The Netherlands

The two-component colR/S system of *Pseudomonas fluorescens* WCS365 plays a role in rhizosphere competence through maintaining the structure and function of the outer membrane

Weert, S. de; Dekkers, L.C.; Bitter, W.; Tuinman, S.; Wijfjes, A.H.M.; Boxtel, R. van; Lugtenberg, E.J.J.

Citation

Weert, S. de, Dekkers, L. C., Bitter, W., Tuinman, S., Wijfjes, A. H. M., Boxtel, R. van, & Lugtenberg, E. J. J. (2006). The two-component colR/S system of *Pseudomonas fluorescens* WCS365 plays a role in rhizosphere competence through maintaining the structure and function of the outer membrane. *Fems Microbiology Ecology*, 2006(58), 205-213.
doi:10.1111/j.1574-6941.2006.00158.x

Version: Not Applicable (or Unknown)
License: [Leiden University Non-exclusive license](#)
Downloaded from: <https://hdl.handle.net/1887/44108>

Note: To cite this publication please use the final published version (if applicable).

The two-component *colR/S* system of *Pseudomonas fluorescens* WCS365 plays a role in rhizosphere competence through maintaining the structure and function of the outer membrane

Sandra de Weert¹, Linda C. Dekkers¹, Wilbert Bitter², Sietske Tuinman¹, André H. M. Wijffjes¹, Ria van Boxtel³ & Ben J. J. Lugtenberg¹

¹Institute of Biology, Leiden University, AL Leiden, The Netherlands; ²Department of Medical Microbiology and Infection Control, Vrije Universiteit Medical Center, Amsterdam, The Netherlands; and ³Department of Microbiology, Utrecht University, Utrecht, The Netherlands

Correspondence: Sandra de Weert, IBL, Leiden University, Molecular Microbiology, Wassenaarseweg 64, 2333AL Leiden, The Netherlands. Tel.: +31 715275075; fax: +31 715275088; e-mail: weert@rulbim.leidenuniv.nl

Present address: Linda C. Dekkers, Pharming Archimedesweg 4, 2333 CN Leiden, The Netherlands.

Present address: André H.M. Wijffjes, Service XS, Wassenaarseweg 72, 2333 AL Leiden, The Netherlands.

Received 23 January 2006; revised 22 March 2006; accepted 27 March 2006.
First published online 12 June 2006.

DOI:10.1111/j.1574-6941.2006.00158.x

Editor: Michael Wagner

Keywords

biological control; root colonization; two-component system; outer-membrane structure.

Introduction

Decreases in crop yields are a world-wide problem. To solve this problem is not only of economical importance but it is also important for the environment. An alternative for chemical pesticides can be the use of beneficial microorganisms. To understand the molecular basis of the control of pathogens by beneficial bacteria it is necessary to study traits essential for biocontrol. Efficient colonization of root systems by beneficial microorganisms is one of the most important traits necessary to establish microbiological control (Chin-A-Woeng *et al.*, 2000). After transposon mutagenesis some genes and traits involved in root colonization have been identified (Lugtenberg *et al.*, 2001). For example, mutant PCL1210, a transposon mutant of the efficient root colonizing biocontrol strain *Pseudomonas fluorescens* WCS365

Abstract

Pseudomonas fluorescens strain PCL1210, a competitive tomato root tip colonization mutant of the efficient root colonizing wild type strain WCS365, is impaired in the two-component sensor-response regulator system ColR/ColS. Here we show that a putative methyltransferase/*wapQ* operon is located downstream of *colR/colS* and that this operon is regulated by ColR/ColS. Since *wapQ* encodes a putative lipopolysaccharide (LPS) phosphatase, the possibility was studied that the integrity of the outer membrane of PCL1210 was altered. Indeed, it was shown that mutant PCL1210 is more resistant to various chemically unrelated antibiotics which have to pass the outer membrane for their action. In contrast, the mutant is more sensitive to the LPS-binding antibiotic polymyxin B. Mutant PCL1210 loses growth in competition with its wild type when grown in tomato root exudate. Mutants in the methyltransferase/*wapQ* operon are also altered in their outer membrane permeability and are defective in competitive tomato root tip colonization. A model for the altered outer membrane of PCL1210 is discussed.

(Dekkers *et al.*, 2000), was initially isolated as a competitive potato root tip colonization mutant (Dekkers *et al.*, 1998). Similarly, the mutant is also impaired in root colonization on various other plants, indicating a broad host range mutation (Dekkers *et al.*, 1998). Three ORFs (open reading frames) are present on the fragment flanking the transposon. The first two ORFs of the complementing region, *colR* and *colS*, show strong homology with known members of two-component regulatory systems (Dekkers *et al.*, 1998). The third one encodes a methyltransferase. Two-component systems, of which more than 50 different sets are presumed to be present in all bacterial species (Rodrigue *et al.*, 2000), enable bacterial cells to respond adequately to a large variety of environmental stimuli. These adaptive responses range from very fast changes necessary for a chemotactic response (Swanson *et al.*, 1994) to changes that in the end lead to the

production of secondary metabolites (Laville *et al.*, 1992). The mutation in mutant PCL1210 is thought to lead to a decreased responsiveness towards the rhizosphere environment that eventually leads to impaired competitive colonization fitness (Dekkers *et al.*, 1998). Here we report that further sequencing of DNA downstream *colR* and *colS* revealed the methyltransferase/*wapQ* operon which appears to be regulated by the *colR/S* system. Further studies on the methyltransferase/*wapQ* operon suggest involvement in the fine-tuning of the permeability function of the outer membrane. A possible model is proposed and discussed in this paper.

Materials and methods

Bacterial strains and growth conditions

Overnight cultures of *P. fluorescens* WCS365 and mutant derivatives were grown at 28 °C in liquid King's medium B (KB; King *et al.*, 1954) under vigorous aeration or on solidified medium containing 1.8% agar. When appropriate, the media were supplemented with kanamycin or tetracycline in final concentrations of 50 and 80 µg mL⁻¹, respectively. To distinguish between *P. fluorescens* WCS365 (white/yellow colonies) and mutant PCL1210 (blue colonies), 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) was used in a final concentration of 40 µg mL⁻¹ as an indicator for β-galactosidase activity.

Antibiotic sensitivity

For experiments in which antibiotic resistance was measured, 0.1 mL of an overnight culture of PCL1210 or WCS365 in KB was diluted into 3 mL of KB top agar (0.6%). The mixture was poured onto the surface of KB plates and was allowed to solidify. Filter paper discs with a diameter of 6 mm (Filter paper GB002, Schleicher and Schuell, Dassel, Germany) were soaked in solutions of various antibiotics and placed on the agar plates. Antibiotic concentrations in the solutions were spectomycin, 100 mg mL⁻¹; tetracycline, 8 mg mL⁻¹; rifampicin, 2 mg mL⁻¹; chloramphenicol, 10 mg mL⁻¹; and streptomycin, 500 mg mL⁻¹. After overnight growth at 28 °C, sensitivity towards the antibiotics was measured by determining the mean ($n=8$) inhibition zone. The obtained data were analyzed for significance with Student's *t*-test ($P=0.05$), using SPSS software (SPSS Inc., Chicago, IL).

A second method used to determine and quantify sensitivity towards antibiotics was performed by growing bacteria overnight and plating these subsequently on increasing concentrations of the appropriate antibiotic.

Sensitivity towards paraquat, a superoxide-generating agent, was measured by inoculating 50 µL of an overnight culture into 5 mL of liquid KB supplemented with different

concentrations of paraquat, followed by overnight growth at 28 °C and measurement of the OD_{620 nm}.

Polymyxin B sensitivity was determined using overnight cells grown in BM2 succinate medium (Gilleland Jr. *et al.*, 1974) supplemented with 0.5 mM MgSO₄. Cells were diluted 100-fold in the same medium as was used for growth in overnight culture except that it was supplemented with different concentrations of polymyxin B. After growth overnight at 28 °C the OD_{620 nm} was measured.

Growth in competition

Competitive growth on root exudate or putrescine (5 mM) as the sole carbon source was performed using overnight grown cells of mutant PCL1210 and wild type WCS365 in liquid basic medium (BM; Lugtenberg *et al.*, 1999). Cells were washed with sterile phosphate-buffered saline (PBS) (Hoffland *et al.*, 1989) and subsequently diluted in BM containing root exudate or putrescine to a final OD_{620 nm} of 0.1. Equal volumes of these cultures were mixed. The mixture was allowed to grow overnight at 28 °C and subsequently diluted again 1000-fold in fresh root exudate or putrescine. This procedure was repeated twice. The ratio between the wild type and the mutant cells was determined at various time intervals by plating diluted samples on solid KB medium supplemented with or without kanamycin.

General DNA techniques

All general DNA techniques, including restriction enzyme digestion, ligations, plasmid isolations, transformation of *Escherichia coli* and nucleotide sequencing were performed as described by Sambrook & Russel (2001).

Using oMP795 (5'-ACCGACCAggggTTCATCCg-3') and oMP796 (5'-gCATgCCATTgATgggggCC-3'), a PCR fragment of the methyltransferase was obtained and cloned to pGEM-T-easy which resulted in pMP5576 (Table 1). After digestion with *SphI* the fragment was cloned into pML103, resulting in pMP5579 (Table 1).

To construct a mutant in the WCS365 gene homologous to the methyltransferase, PCR was performed on genomic DNA of WCS365, using primers oMP747 (5'-CCgAgAAATACgACgAgCagC-3') and oMP748 (5'-TgAATACgAAACCCCTgCCTgC-3'). The resulting PCR fragment was cloned into pGEM-T-easy (= pMP5569) and subsequently cloned, after *EcoRI* digestion, to pMP5285, resulting in plasmid pMP5571 (Table 1). Electroporation to WCS365 resulted in mutant PCL1812 mutated in the gene homologues to a methyltransferase (Table 1).

For the construction of a mutant in the *wapQ* homologue, PCR was performed using oMP751 (5'-CgATTCTT-CACgACgACTTCg-3') and oMP752 (5'-gCTgCCAAACgCSgTCTgYTAA-3'). The obtained fragment was cloned into pGEM-T-easy, resulting in pMP5581 (Table 1). After

Table 1. Bacterial strains and plasmids used

Strains and plasmids	Relevant characteristics	Reference or source
<i>Pseudomonas fluorescens</i>		
WCS365	Wild type biocontrol strain, efficient competitive coloniser of tomato, potato and wheat roots	Schippers <i>et al.</i> (1987); Dekkers <i>et al.</i> (1998)
PCL1285	Km ^r derivative of WCS365 not impaired in competitive root colonisation on tomato and grass; Km ^r	de Weert <i>et al.</i> , (2004a)
PCL1210	Tn5 <i>lacZ colS</i> derivative of WCS365, impaired in competitive tomato root tip colonisation; Km ^r	Dekkers <i>et al.</i> (1998)
PCL1251	Newly generated mutant with an insertion in the <i>colR</i> and <i>colS</i> genes; Tc ^r	Dekkers <i>et al.</i> (1998)
PCL1812	Homologous recombinant in a methyltransferase homologue of WCS365 impaired in competitive tomato root tip colonisation; Km ^r	This study
PCL1823	Homologous recombinant in <i>wapQ</i> homologue of WCS365 impaired in competitive tomato root tip colonisation; Km ^r	This study
PCL1830	WCS365 containing plasmid pMP4068	This study
PCL1831	PCL1210 containing plasmid pMP4068	This study
<i>Escherichia coli</i>		
DH5 α	Used for propagation of plasmids	Hanahan (1983)
XI1blue	Used for transformation and propagation of plasmids	Stratagene, La Jolla, CA
Plasmids		
pGEM-T-easy	Cloning vector for <i>Taq</i> -amplified PCR products; Ap ^r	Promega
pML103	<i>lacZ</i> -fusion broad-host-range vector for Gram negatives	Labes <i>et al.</i> (1990)
pMMB67HE	pMMB66HE Δ [polylinker M13mp18], Ω [polylinker M13mp18]	Furste <i>et al.</i> (1986)
pBBR5MCS5	Broad-host-range cloning vector	Kovach <i>et al.</i> (1995)
pMP4068	pBBR5MCS5 derivative containing the <i>bla</i> gene of pMMB67HE; Gm ^r	This study
pMP5285	pME3049 Voisard <i>et al.</i> (1994) derivative (suicide plasmid for <i>Pseudomonas</i> spp.) used for homologous recombination; Km ^r , Cb ^r	Kuiper <i>et al.</i> (2001)
pMP5569	pGEM derivative containing PCR fragment of a methyltransferase; Cb ^r	This study
pMP5571	pMP5285 containing PCR fragment of methyltransferase of pMP5569; Km ^r	This study
pMP5576	pGEM derivative containing PCR fragment of promoter region methyltransferase; Cb ^r	This study
pMP5579	pML103 derivative containing promoter fragment of methyltransferase from pMP5576; Gm ^r	This study
pMP5580	pML103 derivative containing promoter fragment of methyltransferase from pMP5576 in the wrong orientation; Gm ^r	This study
pMP5581	pGEM derivative containing PCR fragment of <i>wapQ</i> ; Cb ^r	This study
pMP5582	pMP5285 containing PCR fragment of <i>wapQ</i> of pMP5581; Km ^r	This study

digestion with *EcoRI*, the obtained fragment was cloned into pMP5285 resulting in pMP5582, (Table 1) which was subsequently electroporated to WCS365 cells.

Analysis of membrane protein and lipopolysaccharide patterns

Analysis of cell envelope protein patterns (Lugtenberg *et al.*, 1975), and analysis of lipopolysaccharide (LPS) ladder patterns (de Weger *et al.*, 1987) by SDS-polyacrylamide gel electrophoresis was performed as described previously (Goldman & Leive, 1980; Palva & Makela, 1980). Cell envelope protein patterns were analyzed after growth of cells in KB and of cells in BM2 medium supplemented with low (0.02 mM) and high (0.5 mM) Mg²⁺ concentrations. Proteins that were tightly associated with the peptidoglycan were isolated as described by Lugtenberg *et al.* (1977). Cell

envelopes that were treated with trypsin were prepared as described previously (Overbeeke & Lugtenberg, 1980).

Rate of permeation of ampicillin through the outer membrane

The rate of permeation of the β -lactam antibiotic ampicillin through the outer membrane of whole cells was measured using a method originally described by Zimmermann & Rosselet (1977) and modified by Overbeeke & Lugtenberg (1982). Briefly, after overnight growth in KB medium, cells were spun down and washed with buffer A (10 mM HEPES-5 mM MgCl₂, pH 7.0) and set to an OD_{660 nm} value of 0.1. Subsequently the assay was started by the addition of different β -lactam antibiotics at a concentration of 8 mM. After 15 min the reaction was stopped by the addition of freshly prepared stop-mix [containing 8 mM I₂, 0.32 M KI, 2 M HAc, 1 M Na₂WO₄ and 2% (w/v) starch]. After an

additional incubation of 20 min for the colour development, the absorbance of the solution was measured at an optical density of 623 nm.

Competitive tomato root tip colonization assays in a gnotobiotic sand system

Competitive root tip colonization assays were performed as described by Simons *et al.* (1996) using the gnotobiotic system containing sterile quartz sand to which 10% (v/w) PNS (plant nutrient solution) (Hoffland *et al.*, 1989) was added to moisten the sand. For colonization experiments, sterile germinated tomato (*Lycopersicon esculentum* Mill. cv. Carmello; Syngenta, Enkhuizen, the Netherlands) seedlings were inoculated with a mixture of parental strain *P. fluorescens* WCS365 and one of its mutants in a 1:1 ratio. The seedlings were placed in the tubes and allowed to grow in a climate-controlled growth chamber at 18 °C, 70% relative humidity, and 16 h of daylight. After 7 days, root tips (1 cm in length) were isolated with adhering sand, and bacteria were removed from the root parts by shaking in 1.0 mL PBS. The CFU of parental and mutant cells were determined by plating dilutions on solidified KB medium and on the same medium supplemented with kanamycin. The detection limit is 100 CFU mL⁻¹.

All results were statistically analyzed using the nonparametric Wilcoxon–Mann–Whitney test (Sokal & Rohlf, 1981). To avoid log 0 cases, calculations were carried out using log (CFU + 1) cm⁻¹ root tip.

β-galactosidase assay

Cells were grown in root exudate or BM medium with 5 mM putrescine as the sole carbon source at 28 °C. β-galactosidase assays were performed as described by Miller (1972).

Results

Characterization of genes downstream *colR/colS*

Mutant PCL1210 was obtained as a result of the screening of a Tn5lacZ mutant bank of the good root colonizer *P. fluorescens* WCS365 (Dekkers *et al.*, 1998). The transposon was found to be inserted in the *colS* gene of a *colR/colS* two-component regulatory system. Further sequencing of the DNA located downstream of *colR/S* revealed two open reading frames (genbank accession no. Y09798). The first open reading frame, *orf222*, shows 80% identity and 87% similarity at the amino acid level to a SAM-dependent methyltransferase of *P. fluorescens* PfO1 (Genebank accession no. ZP0264083). The predicted amino acid sequence of the second ORF shows significant homology with the KDO, lipopolysaccharide kinase family (pfam06293), such as *inaA* of *E. coli* (Carlson *et al.*, 1984; White *et al.*, 1992) and the

putative heptose kinase *wapQ* of *Pseudomonas aeruginosa* (Walsh *et al.*, 2000). Upstream of the methyltransferase two putative σ70 promoters could be identified, of which the first one is located at bp 2936–18 bp–2912 (-10- -35) and the second one at bp 2926–16 bp–2940 (-10- -35). Both ORFs are preceded by a Shine Dalgarno sequence that is correctly spaced from both start codons. As no putative promoter sequence could be identified between the methyltransferase and *wapQ*, they probably comprise one transcriptional unit in *P. fluorescens* WCS365 (Fig. 1). No significant homology could be identified further downstream of *wapQ*. In *P. aeruginosa* PAO1 a similar highly conserved gene cluster containing five genes can be identified (PA4377–PA4381). In contrast to the four genes we found that a very small gene, PA4377, is present in *P. aeruginosa* PAO1 (<http://www.pseudomonas.com/>) downstream of *WapQ*.

ColR/colS regulates the methyltransferase/*wapQ* operon

It could be possible that the ColR/S system is regulating the downstream *orf222-inaA/wapQ* operon. To test this possibility, the promoter of the methyltransferase gene was cloned in front of a promoterless *lacZ* gene in vector pML103 (Labes *et al.*, 1990), resulting in pMP5579. As a control, a plasmid in which the promoter of the methyltransferase is present in the wrong orientation was constructed (pMP5580, Table 1). Subsequent β-galactosidase activity assays showed that pMP5579 indeed showed promoter activity, whereas the control construct did not (Fig. 2). Moreover, when cells were grown in tomato root exudate or BM containing 5 mM of putrescine, promoter expression in the *colR/colS* mutant, PCL1251, was significantly decreased, relative to that in the wild type (Fig. 2). This result shows that the methyltransferase/*wapQ* operon belongs to the regulon of the ColR/ColS two-component system.

Outer membrane integrity

The *in silico* analysis of *wapQ* suggests that the encoded protein could play a role in LPS modification

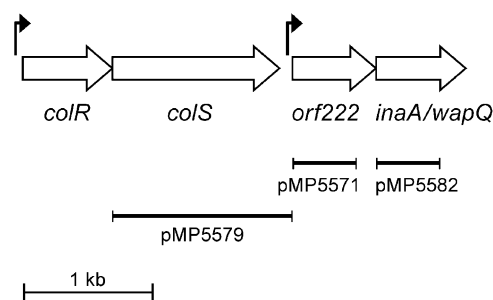


Fig. 1. Map of *colR/colS* and methyltransferase/*wapQ* operon from *Pseudomonas fluorescens* WCS365.

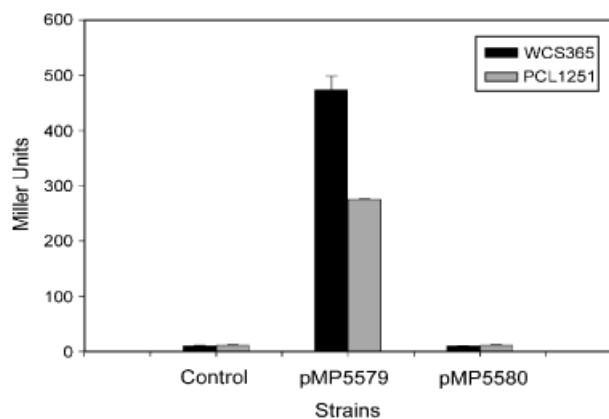


Fig. 2. Promoter activity of methyltransferase in wild type strain WCS365 (dark bars) and *colR/colS* mutant PCL1251 (grey bars). Plasmid pMP5579 contains the promoter of methyltransferase in the correct orientation in front of *lacZ*. Plasmid pMP5580 contains the promoter in the wrong orientation. Strains were grown on BM with 5 mM putrescine as the sole carbon source.

(phosphorylation) and therefore in outer membrane functioning. Since the *ColR/ColS* proteins (*co*)regulate *wapQ*, we tested the effect of the *colR/colS* mutation on a number of outer membrane processes. No differences were observed between the parental strain and mutant, PCL1210, concerning the LPS ladder patterns or cell envelope protein patterns (data not shown), however, phosphorylation and/or methylation differences are also not expected to change the electrophoretic mobility of a large molecule such as LPS. Subsequently, the sensitivity towards a wide range of antibiotics was tested on plates on which filter paper discs soaked in various solutions of these compounds were placed. This analysis showed that mutant PCL1210 was slightly but significantly more resistant towards the antibiotics streptomycin (Fig. 3), rifampicin, spectinomycin and tetracycline (data not shown). Since polymyxin B binds to

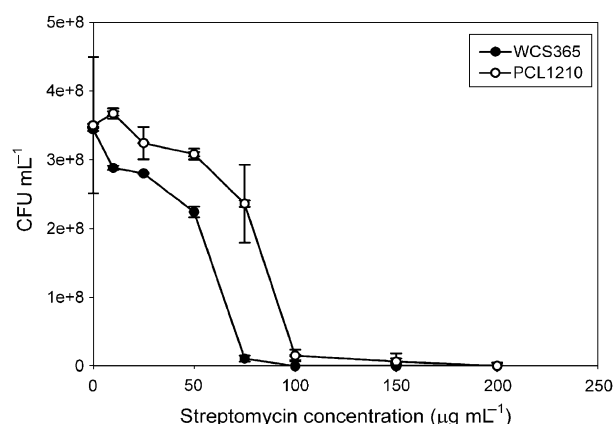


Fig. 3. Sensitivity of *Pseudomonas fluorescens* WCS365 (●) and *colS* mutant PCL1210 (○) towards streptomycin.

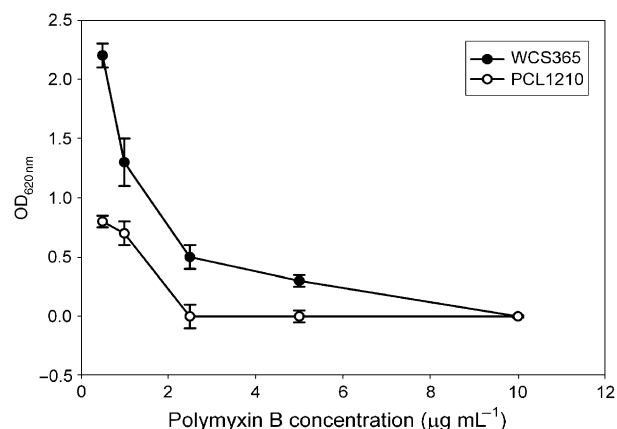


Fig. 4. Growth of *Pseudomonas fluorescens* WCS365 (●) and *colS* mutant PCL1210 (○) after overnight growth in BM2 medium supplemented with 0.5 mM Mg²⁺ and with various concentrations of polymyxin B.

LPS (Nikaido & Vaara, 1987), the *colS/colR* mutant could show changed sensitivity towards this compound. The observation that the growth of this mutant is more sensitive to polymyxin B than the growth of parental strain is (Fig. 4) supports this notion. To study whether the increased sensitivity of the PCL1210 was due to the effect on the methyltransferase/*wapQ* expression, we also constructed mutants in both these genes. For the construction of a mutant in the *wapQ* homologue of WCS365, the forward primer was designed based on WCS365 sequence while the reverse primer was designed based on an alignment of *wapQ* homologues of *P. aeruginosa* and *Pseudomonas putida*. The resulting mutant is referred to as PCL1823 (Table 1). The mutants were not impaired in growth rate when grown in KB medium (data not shown). Indeed, both these mutants showed similar sensitivity results (data not shown). In addition, mutant PCL1210 appeared to be more resistant towards paraquat (Fig. 5).

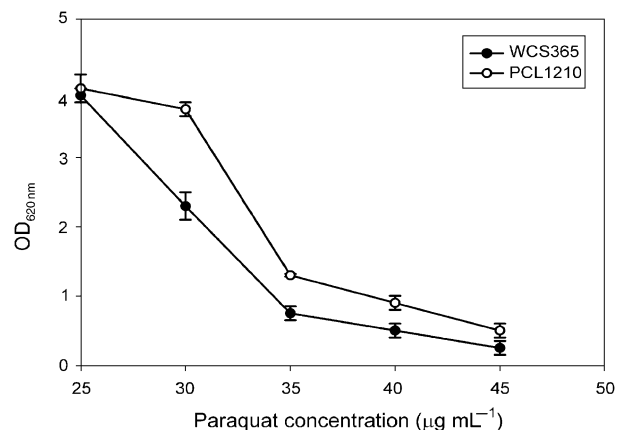


Fig. 5. Growth of wild type *Pseudomonas fluorescens* WCS365 (●) and *colS* mutant PCL1210 (○) after overnight growth in KB medium supplemented with various concentrations of the superoxide forming agent paraquat.

Rate of permeation of ampicillin through the outer membrane

To analyze directly whether the outer membrane of the mutant is altered, the rate of permeation of ampicillin through the outer membrane was measured. The β -lactamase present in the periplasmic space will open the β -lactam ring of those ampicillin molecules that have diffused through the outer membrane protein pores after exogenous application of the antibiotic (Pugsley *et al.*, 1987). The *bla* gene of pMMB67HE (Furste *et al.*, 1986), a plasmid which appeared not to be stable in *P. fluorescens*, was cloned as a HindIII fragment into the broad host range vector pBBR5MCS5 (Kovach *et al.*, 1995), selecting for gentamycin resistance. The resulting plasmid pMP4068 (Table 1) was electroporated into strains WCS365 and PCL1210. The respective resulting strains PCL1830 and PCL1831 (Table 1) were used to determine the rate of permeation of ampicillin through the outer membrane. The results of three experiments showed that the β -lactam antibiotic ampicillin is taken up efficiently by the wild type strain, with an average rate of permeation of $6.2 \text{ nmol}^{-1} \text{ min}^{-1} 10^8 \text{ cells}$. In contrast, the relative rate of β -lactam uptake of mutant PCL1210 was only 18% of that of the wild type. This result indicates that the transport of ampicillin through the general porin(s) was severely affected in the mutant.

colS mutant PCL1210 loses growth in competition on root exudate and its main component putrescine

Because differences in outer membrane permeability, such as the uptake of putrescine, were observed, we reasoned that the colonization defect of the *colR/colS* mutant could be due to the slower uptake of root exudates components. There-

fore, a growth competition was performed in BM medium containing 5 mM of the major root exudate component putrescine as the sole carbon source. The results (Fig. 6) show that the mutant loses slowly but gradually in competition from its parental strain. After approximately 30 generations four times more wild type cells are present than mutant cells (Fig. 6).

Competitive colonization of a methyltransferase- and *wapQ* mutant

To test whether the methyltransferase and *wapQ* genes downstream of *colR/colS* are indeed involved in root colonization, new mutants were constructed by homologous recombination. Mutant PCL1812 is mutated in a gene homologous to a methyltransferase gene (Table 1). The competitive colonizing behaviour of *P. fluorescens* WCS365 and its mutants on tomato roots was analyzed in the gnotobiotic sand system as described by Simons *et al.* (1996). Both mutants PCL1812 (methyltransferase) and PCL1823 (*wapQ*) showed a fivefold decrease in root tip colonizing ability (Table 2).

Discussion

For biological control root colonization is an essential trait. If a bacterium is not able to colonize the root system efficiently, its biocontrol activity is decreased or completely absent (Chin-A-Woeng *et al.*, 1998; Kamilova *et al.*, 2005). When root colonization is studied, CFU are determined from the root tip. Distribution of inocula along the root system shows a gradient (Simons *et al.*, 1996). The major differences between a wild type and a colonization mutant are found on the root tip since that part of the root is most difficult to reach (Chin-A-Woeng *et al.*, 1997; de Weert *et al.*,

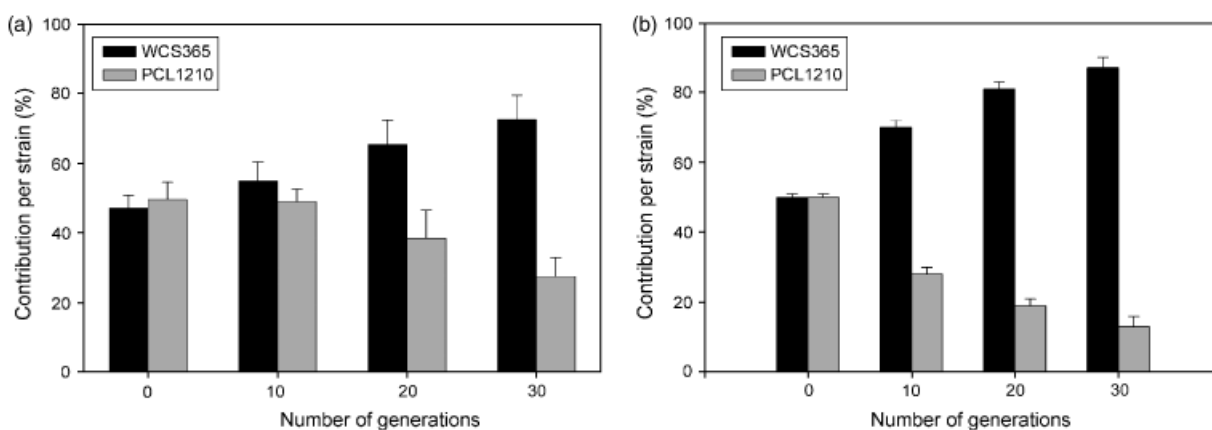


Fig. 6. Competitive growth of *Pseudomonas fluorescens* WCS365 (dark bars) and *colS* mutant PCL1210 (grey bars) in BM with root exudate (a) or putrescine (b) as the sole carbon source. At time point zero individual cultures of both strains were mixed and the number of CFU of each strain was determined. The mixture was diluted 1000-fold, incubated overnight after which the number of CFU of each strain was determined again. This procedure was repeated twice.

Table 2. Competitive tomato root tip colonization of *P. fluorescens* WCS365 and its mutant derivatives in the gnotobiotic system*

Strains	Competitive root tip colonization [log ₁₀ (CFU+1 cm ⁻¹) root tip] [†]	
	Wild type	Mutant
PCL1285 (km ^r parent)	5.6 ± 0.2 ^a	5.6 ± 0.3 ^a
PCL1210 (<i>colS</i>)	5.0 ± 0.3 ^a	2.1 ± 0.4 ^a
PCL1812 (methyltransferase)	5.8 ± 0.1 ^a	5.2 ± 0.1 ^b
PCL1823 (<i>wapQ</i>)	5.5 ± 0.2 ^a	4.9 ± 0.1 ^b

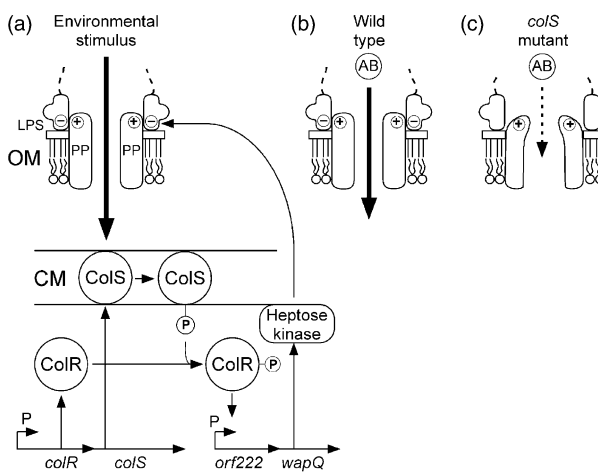
*Seedlings were inoculated with a 1:1 mixture of cells of wild type or mutant strains and placed in the gnotobiotic sand system. Root tips were analyzed after 7 days.

[†]In every experiment 10 seedlings were inoculated and plants were individually processed after growth. When values in the same row are followed by a different letter, they are significantly different at $P=0.05$ on nonparametric multiple comparisons using the Wilcoxon–Mann–Whitney test.

2002). A criterion for a competent root colonizer is that it can efficiently reach the root tip. Unravelling the mechanism behind root colonization is necessary to be able to improve the biocontrol abilities of microorganisms. In this way we can work in an environmentally friendly way on controlling phytopathogens. In a previous study it has been shown that *colS* mutant PCL1210 is impaired in competitive root tip colonization (Dekkers *et al.*, 1998). Here we show that a two-gene operon encoding a methyltransferase and a *wapQ* is present downstream of the *colR/colS* operon (Fig. 1).

In *E. coli* the gene *inaA* is induced by acidification of the external medium. Expression increases with decreasing intracellular pH and with decreasing temperature. In general, *inaA* expression correlated with a decrease in intracellular pH (White *et al.*, 1992). In *E. coli* *inaA* is regulated by both the *soxRS* system, which regulates superoxide stress response, and the MAR system, which regulates multiple antibiotic resistance (Rosner & Slonczewski, 1994).

The *wapQ* gene of *P. aeruginosa* encodes a putative heptose kinase. LPS in the outer membrane contain several heptoses, which are generally phosphorylated. However, the extent of LPS modification is known to vary. For instance, *P. fluorescens* LPS is more heavily phosphorylated at higher temperatures and this modification is probably affecting the pore size of the main porin of *P. fluorescens* OprF (El Hamel *et al.*, 2000). LPS is known to be strongly associated with bacterial porins of *P. aeruginosa* and *E. coli*. It is among others involved in the channel activity of some porins. The negatively charged phosphates on heptose are assumed to contribute to stabilization of the outer membrane structure (Lugtenberg & van Alphen, 1983). This notion, together with the finding that *colS* mutant PCL1210 is more resistant to various chemically unrelated antibiotics, suggested to us a model (Fig. 7) in which passive diffusion of these molecules through the outer membrane pore protein, which interacts

**Fig. 7.** Proposed working model for maintaining the structure and function of the outer membrane.

with LPS (Lugtenberg & van Alphen, 1983) is less efficient in the mutant.

As two-component systems often regulate genes in their close proximity (Swanson *et al.*, 1994), we tested whether *colR/colS* regulates the expression of the methyltransferase/*wapQ* operon. This appeared to be the case although substantial expression still occurs in the *ColR/ColS* mutant (Fig. 2). Because *inaA* expression in *E. coli* is regulated by at least two regulatory systems, *soxRS* and MAR (Rosner & Slonczewski, 1994), a similar complex regulation may occur in *Pseudomonas*. This would explain the high residual activity of the methyltransferase/*wapQ* promoter in a *colR/colS* background (Fig. 2). The proposal that *colR/colS* is required for optimal expression of the methyltransferase/*wapQ* operon is further supported by the observation that like *colS* mutant PCL1210 (Dekkers *et al.*, 1998) both mutants PCL1812 (methyltransferase) and *wapQ* mutant PCL1823 are impaired in competitive tomato root tip colonization (Table 2).

The observation that the rate of degradation of ampicillin by periplasmic β -lactamase is 18% of wild type values in mutant PCL1210 shows that the *colS* mutation negatively affects the permeability of the outer membrane. Consistent with this conclusion is the observation that PCL1210 loses competition for root exudate and the major root exudate component (Kuiper *et al.*, 2001; de Weert *et al.*, 2006) putrescine (Fig. 6). The increased sensitivity of PCL1210 for the antibiotic polymyxin B (Fig. 4), which attacks the outer membrane from the outside by binding to LPS (Nikaido & Vaara, 1987), indicates that, in the mutant, LPS molecules have indeed changed (Ernst *et al.*, 1999). This observation is consistent with a decrease of phosphorylation of heptose in the inner core region of LPS caused by a decreased activity of the WapQ heptose kinase as suggested by the model. It is essential that phosphate residues are present at two locations in the inner core-lipid A region per LPS molecule in wild

type cells (Wilkinson, 1981; Walsh *et al.*, 2000), however the third phosphorylation site of heptose I residue is not essential in *P. aeruginosa* (Walsh *et al.*, 2000).

We propose the following working model. The ColS sensor of the ColR/ColS two-component system is activated by an unknown environmental stimulus. As a consequence ColS is autophosphorylated and subsequently transfers its phosphate residue to ColR. Phosphorylated ColR activates the methyltransferase/*wapQ* operon. The *wapQ* product is homologous to a *P. aeruginosa* heptose kinase responsible for phosphorylating heptose I in the LPS core region (Walsh *et al.*, 2000) The resulting negatively charged phosphorylated LPS is thought to interact with a positively charged part of the pore protein, thereby contributing to keeping the pore open. Considering the possible function of this gene as a methyltransferase, it is possible that modified methylation of LPS also plays a role in the altered outer membrane properties of *colS* mutant PCL1210. Several antibiotics are able to diffuse through the pore of wild type cells. In cells of *colS* mutant PCL1210 the heptose kinase encoded by *wapQ* is less active. As a consequence, the phosphate residues that help to keep the pore open in wild type cells are absent in mutant cells which therefore have a narrower pore through which antibiotics diffuse with a slower rate. Moreover, the space between the pore protein and the LPS molecule at the outside of the cells becomes wider in the mutant which enables polymyxin B to bind to LPS more efficiently. The narrower pore opening in mutant cells makes these cells less efficient than those of the wild type in competing for the low nutrient levels in the rhizosphere. Consequently, the latter cells lose in competitive tomato root tip colonization.

In conclusion, the obtained results support the model described above and presented in Fig. 7 implying that the ColR/ColS regulatory system is required for optimal expression of the methyltransferase/*wapQ* operon and that *wapQ* encodes a heptose kinase. Under-phosphorylation of heptose causes a narrowing of the protein pores in the outer membrane which results in a higher resistance towards several antibiotics and a poorer uptake of the major root exudate component putrescine when presented in a low concentration. Considering the possible function of the downstream gene as a methyltransferase, it is possible that modified methylation of LPS also plays a role in the altered outer membrane properties of *colS* mutant PCL1210. All this leads on the tomato root to a decreased ability of the mutant, after mixed inoculation of the seed, to reach the root tip in competition with the wild type.

References

Carlson J, Fuchs JA & Messing J (1984) Primary structure of the *Escherichia coli* ribonucleoside diphosphate reductase operon. *Proc Natl Acad Sci USA* **81**: 4294–4297.

- Chin-A-Woeng TFC, de Priester W, van der Bij AJ & Lugtenberg BJJ (1997) Description of the colonization of a gnotobiotic tomato rhizosphere by *Pseudomonas fluorescens* biocontrol strain WCS365 using scanning electron microscopy. *Mol Plant-Microbe Interact* **10**: 79–86.
- Chin-A-Woeng TFC, Bloemberg GV, van der Bij AJ, *et al.* (1998) Biocontrol by phenazine-1-carboxamide-producing *Pseudomonas chlororaphis* PCL1391 of tomato root rot caused by *Fusarium oxysporum* f. sp. *radicis lycopersici*. *Mol Plant-Microbe Interact* **11**: 1069–1077.
- Chin-A-Woeng TFC, Bloemberg GV, Mulders IHM, Dekkers LC & Lugtenberg BJJ (2000) Root colonization by phenazine-1-carboxamide-producing bacterium *Pseudomonas chlororaphis* PCL1391 is essential for biocontrol of tomato foot and root rot. *Mol Plant-Microbe Interact* **13**: 1340–1345.
- Dekkers LC, Bloemendaal CJ, de Weger LA, Wijffelman CA, Spaik HP & Lugtenberg BJJ (1998) A two-component system plays an important role in the root-colonizing ability of *Pseudomonas fluorescens* strain WCS365. *Mol Plant-Microbe Interact* **11**: 45–56.
- Dekkers LC, Mulders IHM, Phoelich CC, Chin-A-Woeng TFC, Wijffels AHM & Lugtenberg BJJ (2000) The *sss* colonization gene of the tomato-*Fusarium oxysporum* f. sp. *radicis lycopersici* biocontrol strain *Pseudomonas fluorescens* WCS365 can improve root colonization of other wild-type *Pseudomonas* spp. Bacteria. *Mol Plant-Microbe Interact* **13**: 1177–1183.
- de Weert S, Vermeiren H, Mulders IHM, Kuiper I, Hendrickx N, Bloemberg GV, Vanderleyden J, de Mot R & Lugtenberg BJJ (2002) Flagella-driven chemotaxis towards exudate components is an important trait for tomato root colonization by *Pseudomonas fluorescens*. *Mol Plant-Microbe Interact* **15**: 1173–1180.
- de Weert S, Dekkers LC, Kuiper I, Bloemberg GV & Lugtenberg BJJ (2004a) Generation of enhanced competitive root tip colonizing *Pseudomonas* bacteria through accelerated evolution. *J Bacteriol* **186**: 3153–3159.
- de Weert S, Kuiper I, Kamilova FD, *et al.* (2006) Role of competitive root tip colonisation in the biological control of tomato foot and root rot. *Biological Control of Fruit and Vegetables Diseases*. Kluwer Academic Publishers, Dordrecht, The Netherlands, in press.
- de Weger LA, Jann B, Jann K & Lugtenberg BJJ (1987) Lipopolysaccharides of *Pseudomonas* spp. that stimulate plant growth: composition and use for strain identification. *J Bacteriol* **169**: 1441–1446.
- El Hamel C, Freulet MA, Jaquinod M, De E, Molle G & Orange N (2000) Involvement of the C-terminal part of *Pseudomonas fluorescens* OprF in the modulation of its pore-forming properties. *Biochim Biophys Acta* **1509**: 237–244.
- Ernst RK, Yi EC, Guo L, Lim KB, Burns JL, Hackett M & Miller SI (1999) Specific lipopolysaccharide found in cystic fibrosis airway *Pseudomonas aeruginosa*. *Science* **286**: 1561–1565.
- Furste JP, Pansegrau W, Frank R, Blocker H, Scholz P, Bagdasarian M & Lanka E (1986) Molecular cloning of the plasmid RP4 primase region in a multi-host-range *tacP* expression vector. *Gene* **48**: 119–131.
- Gilleland HE Jr, Stinnett JD & Eagon RG (1974) Ultrastructural and chemical alteration of the cell envelope of *Pseudomonas*

- aeruginosa*, associated with resistance to ethylene-di-amine-tetra-acetate resulting from growth in a Mg^{2+} -deficient medium. *J Bacteriol* **117**: 302–311.
- Goldman RC & Leive L (1980) Heterogeneity of antigenic-side-chain length in lipopolysaccharide from *Escherichia coli* 0111 and *Salmonella typhimurium* LT2. *Eur J Biochem* **107**: 145–153.
- Hanahan D (1983) Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* **166**: 557–580.
- Hoffland E, Findenegg GR & Nelemans JA (1989) Solubilization of rock phosphate by rape. *Plant Soil* **113**: 161–165.
- Kamilova F, Validov S, Azarova T, Mulders I & Lugtenberg BJJ (2005) Enrichment for enhanced competitive plant root tip colonizers selects for a new class of biocontrol bacteria. *Environ Microbiol* **7**: 1809–1817.
- King EO, Ward MK & Raney DE (1954) Two simple media for the demonstration of pyocyanin and fluorescin. *J Lab Clin Med* **44**: 301–307.
- Kovach ME, Elzer PH, Hill DS, Robertson GT, Farris MA, Roop RM & Peterson KM (1995) Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* **166**: 175–176.
- Kuiper I, Bloemberg GV, Noreen GV, Thomas-Oates JE & Lugtenberg BJJ (2001) Increased uptake of putrescine in the rhizosphere inhibits competitive root colonization by *Pseudomonas fluorescens* strain WCS365. *Mol Plant-Microbe Interact* **14**: 1096–1104.
- Labes M, Puhler A & Simon R (1990) A new family of RSF1010-derived expression and lac-fusion broad-host-range vectors for gram-negative bacteria. *Gene* **89**: 37–46.
- Laville J, Voisard C, Keel C, Maurhofer M, Defago G & Haas D (1992) Global control in *Pseudomonas fluorescens* mediating antibiotic synthesis and suppression of black root rot of tobacco. *Proc Natl Acad Sci USA* **89**: 1562–1566.
- Lugtenberg BJJ & van Alphen L (1983) Molecular architecture and functioning of the outer-membrane of *Escherichia coli* and other gram-negative bacteria. *Biochim Biophys Acta* **737**: 51–115.
- Lugtenberg BJJ, Meyers J, Peters R, van der Hoek P & van Alphen L (1975) Electrophoretic resolution of the major outer membrane protein of *Escherichia coli* K12 into four bands. *FEBS* **51**: 115.
- Lugtenberg BJJ, Bronstein H, van selm N & Peters R (1977) Peptido-glycan associated outer membrane proteins in gram-negative bacteria. *Biochim Biophys Acta* **571**: 571–578.
- Lugtenberg BJJ, Kravchenko LV & Simons M (1999) Tomato seed and root exudate sugars: composition, utilization by *Pseudomonas* biocontrol strains and role in rhizosphere colonization. *Environ Microbiol* **1**: 439–446.
- Lugtenberg BJJ, Dekkers LC & Bloemberg GV (2001) Molecular determinations of rhizosphere colonization by *Pseudomonas*. *Annu Rev Phytopathol* **39**: 461–490.
- Miller JH (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor Lab, New York.
- Nikaido H & Vaara M (1987) Outer membrane. *Escherichia coli* and *Salmonella typhimurium* (Ingraham JL, Brooks Low K, Magasanik B, Schaechter M & Umbarger HE, eds), pp. 1–22. American Society for Microbiology, Washington.
- Overbeeke N & Lugtenberg B (1980) Major outer membrane proteins of *Escherichia coli* strains of human origin. *J Gen Microbiol* **121**: 373–380.
- Overbeeke N & Lugtenberg B (1982) Recognition site for phosphorus-containing compounds and other negatively charged solutes on the PhoE protein pore of the outer membrane of *Escherichia coli* K12. *Eur J Biochem* **126**: 113–118.
- Palva ET & Makela PH (1980) Lipopolysaccharide heterogeneity in *Salmonella typhimurium* analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. *Eur J Biochem* **107**: 137–143.
- Pugsley AP, Zimmerman W & Wehrli W (1987) Highly efficient uptake of a rifamycin derivative via the FhuA-TonB-dependent uptake route in *Escherichia coli*. *J Gen Microbiol* **133**: 3505–3511.
- Rodrigue A, Quentin Y, Lazdunski A, Mejean V & Foglino M (2000) Two-component systems in *Pseudomonas aeruginosa*: why so many? *Trends Microbiol* **8**: 498–504.
- Rosner JL & Slonczewski JL (1994) Dual regulation of *inaA* by the multiple antibiotic resistance (*mar*) and superoxide (*soxRS*) stress response systems of *Escherichia coli*. *J Bacteriol* **176**: 6262–6269.
- Sambrook J & Russel D (2001) *Molecular Cloning: A Laboratory Manual*. Cold Spring Laboratory Press, Cold Spring Harbor NY, USA.
- Schippers B, Bakker AW & Bakker PAHM (1987) Interactions of deleterious and beneficial rhizosphere microorganisms and the effect of cropping practices. *Annu Rev Phytopathol* **25**: 339–358.
- Simons M, van der Bij AJ, Brand J, de Weger LA, Wijffelman CA & Lugtenberg BJJ (1996) Gnotobiotic system for studying rhizosphere colonization by plant growth-promoting *Pseudomonas* bacteria. *Mol Plant-Microbe Interact* **9**: 600–607.
- Sokal RR & Rohlf FJ (1981) *Biometry*. Freeman, San Francisco.
- Swanson RV, Alex LA & Simon MI (1994) Histidine and aspartate phosphorylation: two-component systems and the limits of homology. *Trends Biochem Sci* **19**: 485–490.
- Voisard C, Bull CT, Keel C, Laville J, Maurhofer M, Schnider U, Defago G & Haas D (1994) Biocontrol of root diseases by *Pseudomonas fluorescens* CHAO: current concepts and experimental approaches. *Molecular Ecology of Rhizosphere Microorganisms* (O’Gara F, Dowling DN & Boesten N, eds), pp. 67–89. VCH Verlagsgesellschaft, Weinheim, Germany.
- Walsh AG, Matewish MJ, Burrows LL, Monteiro MA, Perry MB & Lam JS (2000) Lipopolysaccharide core phosphates are required for viability and intrinsic drug resistance in *Pseudomonas aeruginosa*. *Mol Microbiol* **35**: 718–727.
- White S, Tuttle FE, Blankenhorn D, Dosch DC & Slonczewski JL (1992) pH dependence and gene structure of *inaA* in *Escherichia coli*. *J Bacteriol* **174**: 1537–1543.
- Wilkinson SG (1981) ³¹P N.m.r. evidence for the presence of triphosphate residues in lipopolysaccharides from *Pseudomonas aeruginosa*. *Biochem J* **199**: 833–835.
- Zimmermann W & Rosselet A (1977) Function of the outer membrane of *Escherichia coli* as a permeability barrier to beta-lactam antibiotics. *Antimicrob Agents Chemother* **12**: 368–372.