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Deletion of the \textit{yiaMNO} transporter genes affects the growth characteristics of \textit{Escherichia coli} K-12

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Binding-protein-dependent secondary transporters make up a unique transport protein family. They use a solute-binding protein in proton-motive-force-driven transport. Only a few systems have been functionally analysed. The \textit{yiaMNO} genes of \textit{Escherichia coli} K-12 encode one family member that transports the rare pentose \textit{L}-xylulose. Its physiological role is unknown, since wild-type \textit{E. coli} K-12 does not utilize \textit{L}-xylulose as sole carbon source. Deletion of the \textit{yiaMNO} genes in \textit{E. coli} K-12 strain MC4100 resulted in remarkable changes in the transition from exponential growth to the stationary phase, high-salt survival and biofilm formation.

INTRODUCTION

Prokaryotes employ several classes of transport systems for the uptake of solutes from their environment, which are defined on the basis of their subunit composition and mode of energization (Driessen et al., 2000). The recently discovered binding-protein-dependent secondary (Driessen et al., 1997, 2000), or tripartite ATP-independent periplasmic (TRAP) (Forward et al., 1997; Rabus et al., 1999; Kelly & Thomas, 2001) transporters utilize a solute-binding protein that captures the substrate at the outside of the cell and deliver it to a membrane permease that is made up of two subunits. The large subunit contains 12 putative transmembrane domains (TMDs) and a large cytoplasmic loop between TMD 6 and TMD 7, and thus resembles classical secondary transporters. The small subunit is made up of four putative TMDs. Transport is driven by the proton-motive force (pmf), in contrast to the more familiar binding-protein-dependent ATP-binding cassette (ABC) transporters that are energized by ATP hydrolysis (Driessen et al., 1997, 2000; Forward et al., 1997; Rabus et al., 1999; Kelly & Thomas, 2001; Wyborn et al., 2001).

To date, only a few members of this class of transporters have been functionally described. One member is involved in the sodium- and pmf-dependent uptake of glutamate by \textit{Rhodobacter sphaeroides} (Jacobs et al., 1996). The DctPQM transporter of \textit{Rhodobacter capsulatis} transports the \textit{C}_4- dicarboxylates malate, succinate and fumarate (Forward et al., 1997) and a similar system was found in \textit{Wolinella succinogenes} (Ullmann et al., 2000). More recently, TeaABC of \textit{Halomonas elongata} was shown to transport the compatible solutes ectoine and hydroxyectoine (Grammann et al., 2002). Consequently, these transporters may play roles both in the uptake of carbon sources and in the protection of the cell against non-favourable conditions. The \textit{Escherichia coli} K-12 genome contains one binding-protein-dependent secondary transporter encoded by the \textit{yiaMNO} genes, located in the \textit{yiaKLMNOPQRS} gene cluster. \textit{yiaO} encodes the periplasmic binding protein, while \textit{yiaM} and \textit{yiaN} encode the small and large membrane protein, respectively. Based on identity with the genes encoding TeaABC, YiaMNO has been suggested to be involved in the uptake of osmoprotectants (Ly et al., 2004). However, experimental evidence indicates that YiaMNO catalyses the uptake of the rare pentose \textit{L}-xylulose (\textit{1,3-threo-2-pentulose}) (Plantinga et al., 2004a). \textit{L}-Xylulose is presumably metabolized by the enzymes encoded immediately downstream of the \textit{yiaMNO} genes, since \textit{L}-xylulose transport and metabolism is found only in cells that constitutively express the \textit{yiaK}-\textit{S} gene cluster. This coupling of the proposed carbohydrate transport and metabolism function is conserved in at least 26 bacterial genomes, most of which are human pathogens (Plantinga et al., 2004b). However, \textit{L}-xylulose does not induce expression of these genes and little is known about its utilization by \textit{E. coli} K-12 (Ibáñez et al., 2000; Plantinga et al., 2004a), indicating that \textit{L}-xylulose might not be the sole substrate for the YiaMNO transporter.

This study focuses on the physiological role of the \textit{yiaMNO} genes. We have characterized \textit{E. coli} strain K-12 MC4100, which is widely used in gene expression work (Peters et al.,...
2003), and a ΔyiaMNO derivative thereof. Some striking phenotypic differences between the ΔyiaMNO mutant and parent strain were observed in our growth experiments.

**METHODS**

**Bacterial strains and growth conditions.** All strains that were used in this study are *E. coli* K-12 derivatives: MC4100 (F’ araD139 Δ(argF-lac)U169 rpsL150 relA1 fliB5301 deoC1 ptsF25 rbsB; Casabadian, 1976); ECL1 [HfrC phoA8 relA1 tonA22 T2’ (λ); Lin, 1976]; JA134 (ECL1 lyx+; Sánchez et al., 1994); MG1655 (F’ λ’ ilvG rfb50 rphl; Blattner et al., 1997). The unmarked chromosomal deletion of the *yiaMNO* genes was created in strains MC4100, JA134 and MG1655 as described elsewhere (Plantinga et al., 2004a) and the respective mutants were labelled TP001, TP018 and TP010. Cells were grown aerobically in Luria–Bertani (LB), LB supplemented with 0-5 % (w/v) glucose (LBG) or M63 minimal medium (Miller, 1972) at 37 °C. Growth was monitored by measurements of OD<sub>660</sub>. The number of c.f.u. was determined by plating serial dilutions on LB-agar. Antibiotics were used at 50 μg ml<sup>-1</sup> and 12 μg ml<sup>-1</sup> for ampicillin and tetracyclin, respectively. For high-salt growth experiments, LB and LBG were supplemented with 0.7–1.0 M NaCl or KCl.

**Expression of the yiaM and yiaO genes.** Cells were grown aerobically in LB and LBG, samples were taken at various OD<sub>660</sub> values and total RNA was extracted as described by Plantinga et al. (2004a). RT-PCR was performed on 1 μg total RNA using RT-PCR beads (Amersham Pharmacia Biotech) and primers directed against 474 and 632 bp fragments of the *yiaM* and *secY* genes, respectively (Plantinga et al., 2004a). For Northern Blot analysis, 5 μg total RNA extracted from each sample was run on a 1·2 % (w/v) agarose gel containing 1 % SDS, and the probe was purified using a PCR product isolation procedure (Qiagen) and hybridized overnight with 0·2 μg/ml 32P dCTP using Klenow polymerase (Roche). The probe was purified using a PCR product isolation procedure (Qiagen) and hybridized overnight in 0·5 M sodium phosphate, pH 7·2, 1 % (w/v) blocking reagent (Roche) and 7 % (w/v) SDS at 65 °C. The membrane was washed in 50 mM sodium phosphate, pH 7·2, containing 1 % SDS, and the signal was recorded by autoradiography.

**Promoter induction and β-galactosidase assays.** The 1000 bp upstream region of *yiaM* was cloned via PCR. The forward primer (5’-ATGGGATCCATTTAAAAGGAAAAATATTATG-3’) and the reverse primer (5’-CTTGGATCCATTTGAGGCA-3’) introduced BamHI and XbaI restriction sites, respectively. The fragment was ligated into vector pET401 (Van der Sluis et al., 2002) for propagation and subsequently used as a template for labelling with 32P dCTP using Klenow polymerase (Roche). The probe was purified using a PCR product isolation procedure (Qiagen) and hybridized overnight in 0·5 M sodium phosphate, pH 7·2, 1 % (w/v) blocking reagent (Roche) and 7 % (w/v) SDS at 65 °C. The membrane was washed in 50 mM sodium phosphate, pH 7·2, containing 1 % SDS, and the signal was recorded by autoradiography.

**Spent medium growth experiments.** MC4100 and TP001 cells were grown aerobically in LB or LBG; 50 ml samples were taken at OD<sub>660</sub> values of 0·5, 1·5, 2·0 and 2·5, and harvested (4000 g, 10 min, 4 °C). The supernatant was filtered to remove all cells. Fresh LB or LBG cultures of MC4100 and TP001 were grown to an OD<sub>660</sub> of 0·5, harvested and resuspended in equal volumes of pre-warmed (37 °C) spent medium. Aerobic growth was continued and monitored over time.

**NMR analysis.** MC4100 and TP001 were grown to an OD<sub>660</sub> of 0·5 in a total of 8 l LBG containing 0·9 M NaCl per strain. Cells were harvested and freeze-dried overnight. Ethanol extracts for determination of intracellular solutes were prepared as described elsewhere (Martins & Santos, 1995). 13C-NMR spectra were measured by Dr A. Ramos at the Instituto Tecnologia Quimica e Biologica of the University of Lisbon, Portugal, using a Bruker DRX500 spectrometer as described by Martins & Santos (1995).

**Biofilms.** Cells were plated on LB-agar and grown overnight at 37 °C. Colonies were picked and resuspended in M63 minimal medium and diluted into untreated polystyrene 96-wells plates (Costar) containing M63 0·5 % (w/v) glucose. Plates were incubated at 37 °C for 60 h and biofilm formation was quantified using crystal violet, as described by O’Toole et al. (1999).

**RESULTS**

**Expression of the yiaMNO genes in *E. coli* strain MC4100.** To determine the physiological role of the *yiaMNO* genes, we first determined their expression by RT-PCR. *E. coli* strain MC4100 was grown aerobically in LB or in LB supplemented with 0·5 % (w/v) glucose (Fig. 1a). Under these conditions the cells expressed the yiaM gene during growth in both LB and LBG (Fig. 1b, c). Expression appeared to be growth-phase-dependent and was maximal in the late stationary phase (Fig. 1b, c). Expression was not repressed in the presence of glucose (Fig. 1c). These results were supported by Northern Blotting using the yiaO gene as the hybridization probe, detecting a fragment of 8·2 kb, which is the expected size for the yiaK-S messenger (data not shown; Ibañez et al., 2000).

*E. coli* strain JA134 expresses the yiaK-S gene cluster from one promoter upstream of the yiaK gene (Ibañez et al., 2000). Computational analysis of this region on the *E. coli* K-12 genome, using RegulonDB, identified an additional putative promoter immediately upstream of *yiaM* (Salgado et al., 2001). This putative promoter was translationally fused to a lacZ-reporter gene in vector pBC3 (Meier et al., 1991). This vector, termed pP1900, was transformed into strain MC4100. Heat and cold shock were applied by shifting liquid cultures to 42 and 10 °C, respectively. High salt, sucrose and spent medium (prepared as described below) effects were determined by harvesting the cells and resuspending them in the respective media. Induction by 1-xylose was tested by harvesting the cells and resuspending them in minimal medium containing 0·4 % 1-xylose. Following 30 min of incubation, the cells were harvested and β-galactosidase assays were performed as described by Miller (1992). Values were expressed in Miller units (MU).

**Spent medium growth experiments.** MC4100 and TP001 cells were grown aerobically in LB or LBG; 50 ml samples were taken at
A striking phenotypical difference between mutant and parent strain was observed in growth experiments. When grown aerobically in LBG batch culture, i.e. containing glucose, both strains grew at nearly identical rates during the exponential phase, with the relatively sharp transition from exponential to stationary phase typical for a carbon-limited batch culture (Mason & Egli, 1993). However, for TP001 this transition was delayed, reproducibly yielding a higher final OD$_{660}$ at the stationary phase (Fig. 2). This was supported by c.f.u. numbers (data not shown). This difference in growth was not observed in LB (Fig. 2). When the cells were growing in minimal media supplied either with glucose or other carbon sources, a similar difference in final OD$_{660}$ was observed (data not shown; compounds listed in Plantinga et al., 2004a). These findings suggest that, under specific conditions, the deletion of the three structural genes encoding the YiaMNO transporter affects growth, and in particular the ability of the cells to enter stationary phase.

Interestingly, autoinducer 2 (AI-2), the quorum sensing signal molecule of *E. coli* and a range of other bacteria, is produced under conditions where the effects of the yiaMNO-deletion were observed (Surette & Bassler, 1998; Bassler, 2002; Chen et al., 2002). Deletion of the AI-2-producing enzyme affects growth in a similar fashion (Sperandio et al., 1999). Therefore, we investigated the involvement of the transporter, or the transported substrate(s), in quorum sensing by examining the effect of spent medium on growth. Spent medium taken from MC4100 cultures grown in LB hardly influenced the growth of fresh cultures (Fig. 3, black and white bars). In contrast, addition of LBG spent medium taken at an OD$_{660}$ > 1.5 clearly negatively affected growth (Fig. 3, dark grey and light grey bars). However, no differences in response were observed between MC4100 (Fig. 3, black and dark grey bars) and TP001 (Fig. 3, white and light grey bars). Spent medium prepared from TP001 yielded identical results (data not shown). These findings indicate that if the autoinducer is indeed produced by these strains during growth on LBG, the ability of TP001 to respond to the molecule is not affected by the deletion of the yiaMNO genes. We therefore conclude that YiaMNO is not involved in the uptake of AI-2 or any other compound with a similar function.

Deletion of the YiaMNO genes affects high-salt tolerance

The *H. elongata* TeaABC transporter protects the cell against hyperosmotic conditions (Grammann et al., 2002) and it has been suggested that the YiaMNO transporter may play a similar role in *E. coli* (Ly et al., 2004). Therefore, the ability of strains MC4100 and TP001 to survive hyperosmotic stress was investigated. Following dilution into LBG containing 0.9 M NaCl, which is 10-times the concentration of...
NaCl in LB(G), MC4100 started doubling after a lag time of 1 h 48 min ± 8 min (six independent experiments) and reached a final OD$_{660}$ of about 2 (Fig. 4, black triangles). However, growth of the deletion mutant was delayed at this high salt concentration, starting an additional 1 h 22 min ± 12 min (n=6) after the wild-type strain had resumed growth (Fig. 4, inset). TP001 did reach the same final OD$_{660}$ of about 2 (Fig. 4, white triangles). Strain TP001 did not grow at all at 1 M NaCl (Fig. 4). Similar results were obtained when the cells were grown in the presence of high KCl concentrations, but not in media containing high sucrose (data not shown). This suggests that the YiaMNO transporter may indeed be involved in the accumulation of an osmoprotective compound. To identify a possible substrate involved in this process, we performed $^{13}$C-NMR analysis of whole-cell extracts obtained from both E. coli strains grown to an OD$_{660}$ of 0.5 in LB containing 0.9 M NaCl. However, no differences in total accumulated cellular compounds could be detected (data not shown). The major compatible solute that had been accumulated by both strains was identified as glycine betaine. Growth of both MC4100 and TP001 on M63 minimal medium with glucose as sole carbon source was also negatively affected by NaCl at concentrations above 0.7 M. Again, TP001 showed a delay in recovery from the high-salt challenge (data not shown). In these experiments, insufficient biomass was obtained to allow for $^{13}$C-NMR analysis of whole-cell extracts. However, growth of both strains could be restored to identical levels by the addition of 1 mM of the osmoprotectants glycine betaine, l-proline, potassium glutamate or ectoine (data not shown). In this regard, none of these compatible solutes are substrates for the YiaMNO transporter (Plantinga et al., 2004a).

**Deletion of the yiaMNO genes reduces biofilm formation**

Loss of a 2·4 kb genomic fragment containing the yiaMNO homologues of the Gram-negative bacterium *Ralstonia* sp. TTD14 (Nakatsu et al., 1998) led to changes in bile salt sensitivity and adhesion (Riley et al., 2001). Since the size of the complete deletion was not determined, it remains unknown whether the YiaMNO homologue contributes to this phenotype. Therefore, we investigated whether the ability to attach to surfaces is affected in the yiaMNO mutant of *E. coli*. Biofilms were allowed to form for 60 h in M63 minimal medium in the presence of D-glucose as sole carbon source. As observed before, TP001 reached a higher final OD$_{660}$ compared to MC4100 during growth (data not shown). However, biofilm formation was clearly negatively affected by deletion of the YiaMNO transporter (Fig. 5). This demonstrates that changes in adhesion may indeed be attributed to deletion of the yiaMNO genes.

**DISCUSSION**

An increasing number of whole-genome expression profiles has been published in recent years (e.g. Tao et al., 1999; Arfin et al., 2000).
Deletion of the YiaMNO transporter affects growth

eq 0.05

**Fig. 5.** Biofilm formation by MC4100 and TP001 detected by crystal violet staining of surface-attached biomass. Cells were grown for 60 h in M63 minimal medium in the absence (−) or presence (+) of D-glucose. In the presence of D-glucose both strains form biofilms, but this is reduced in the yiaMNO deletion mutant in comparison to the wild-type strain.

et al., 2000; Arnold et al., 2001; Pomposiello et al., 2001; Wei et al., 2001; Beloin et al., 2004; Ren et al., 2004; Kang et al., 2005). Although a range of physiological conditions have been tested in these studies with various *E. coli* K-12 strains, including the sequenced strain MG1655, no specific pattern of expression of the yiaK-S genes was detected. Expression of the yiaK-S gene cluster has been observed in *E. coli* strain JA134, a derivative of K-12 strain ECL1, following IS-mediated disruption of the yiaJ gene immediately upstream of yiaK (Badia et al., 1998). The disrupted yiaJ gene encodes the repressor of the gene cluster and the event leads to constitutive expression of the yiaK-S genes in strain JA134 (Badia et al., 1998). Activation of this gene cluster had previously been shown to lead to the expression of *L*-xylulose kinase and allowed strain JA134 to grow on *L*-lyxose (Sánchez et al., 1994). However, *L*-lyxose is neither an inducer of yiaK-S expression (Ibañez et al., 2000) nor is it a substrate for the transporter encoded by the yiaMNO genes, which has recently been shown to transport the rare pentose *L*-xylulose (Plantinga et al., 2004a). As strain JA134 lacks the YiaJ repressor of the yiaK-S genes, we cannot exclude the possibility that different regulatory circuits are active in this strain. Thus, the combined available data does not shed light on the physiological role of these genes. The study presented here addresses the physiological function of the yiaMNO genes, encoding a binding-protein-dependent secondary transporter. We made use of *E. coli* K-12 MC4100, a strain widely used in gene expression studies, which expresses the genes in a growth-phase-dependent manner (Fig. 1).

Although expression of the yiaK-S gene cluster in strain JA134 is subject to carbon catabolite repression via the cyclic AMP receptor protein (CRP) (Ibañez et al., 2000), in strain MC4100 expression does not appear to be repressed by glucose (Fig. 1). Interestingly, transcription of the yiaK promoter is highly upregulated in the mouse pathogen *Salmonella enterica* serovar Typhimurium SL1344 during colonization of the caecum of a murine enteritis model, and to a lesser extent in a murine typhoid fever model (C. Rollenhagen and D. Bumann, personal communication). Moreover, the promoter is also active in an *in vitro* model mimicking the conditions in the gut, i.e. low oxygen and increased NaCl concentration (C. Rollenhagen and D. Bumann, personal communication). These recent findings therefore support the notion that the YiaMNO transporter may play a role in scavenging scarce substrates under the limiting conditions encountered by this pathogen inside a eukaryotic host. Indeed, in *E. coli* strain MC4100, we detect the highest expression levels in cells that had been in stationary phase for 24 h (Fig. 1). Since *L*-xylulose does not induce expression of the YiaMNO transporter, while expression does not appear to be repressed by glucose, a major role of this system in carbon source uptake and utilization is not evident at this time.

The loss of the YiaMNO transporter clearly affects growth of strain MC4100, in particular the transition from exponential to stationary growth on LBG (Fig. 2). In the yiaMNO mutant this transition was delayed, yielding a higher final 

OD660 during stationary phase. Strikingly, this may be related to a phenomenon observed in *Ralstonia* sp. TFD14. After 1000 generations of experimental evolution, all evolved populations showed an increased fitness compared to the ancestor strain (Korona et al., 1994) and 71 out of 72 evolved populations had lost the 2·4 kb genomic fragment containing the *Ralstonia* yiaMNO-homologues (Nakatsu et al., 1998). The size of the complete deletion was not determined, but our data now clearly demonstrate that this growth advantage may be attributed to the yiaMNO deletion. The observed increased fitness may be related to a defective quorum sensing circuit, in which the transporter is involved in the uptake of a signalling molecule that negatively affects growth. However, although our data suggest that strain MC4100 produces an 'autoinducer-like’ activity, the YiaMNO transporter is not required for transport of the autoinducer or a precursor thereof (Fig. 3).

In the evolved populations of *Ralstonia* sp. the extracellular polysaccharide (EPS) had disappeared, leading to changes in bile salt sensitivity and adhesion properties (Riley et al., 2001). Although the yiaMNO mutant is clearly more sensitive to high salt, we were unable to identify a possible transported substrate involved in osmoprotection. The major EPS of *E. coli* K-12, colanic acid (Rick & Silver, 1996), is required for development of biofilm architecture (Prigent-Combaret et al., 1999; Danese et al., 2000). It has been shown that deletion of the producing genes delays, but does not abolish, biofilm formation, with nearly identical amounts of surface-attached biomass at times ≧ 45 h (Danese et al., 2000). However, we still observe major differences in biofilm formation after 60 h (Fig. 5), therefore colanic acid biosynthesis most likely is not affected by the yiaMNO deletion.
Nevertheless, the possible (indirect) involvement of the YiaMNO transporter in EPS biosynthesis does provide a basis for future experiments.

The yiaMNO derivatives of two additional strains were used in the biofilm experiments to test whether the observed effects are strain-specific. No effect of the deletion on biofilm formation was found in strain JA134, which, as its parent ECL1, formed significantly higher amounts of biofilm than strain MC4100 in the assay (data not shown). The effect of the deletion may therefore have been obscured in this genetic background (see above). Deletion of the yiaMNO transporter in the sequenced K-12 strain MG1655, yielding strain TP010, did however reduce biofilm formation (data not shown). In this regard, a negative effect similar to that observed in strain TP001 was observed during growth of strain TP010 on LB(G) in the presence of high salt (data not shown). Although significant genomic differences have been detected between both strains (Peters et al., 2003) these do not localize to the region encoding the yiaMNO genes and our findings do not appear to be strain-specific.

**Concluding remarks**

The phenotypic effects we have observed in the yiaMNO mutant of E. coli K-12 strain MC4100 are diverse and the pathways that underlie these phenomena are complex. However, the observations made with *Ralstonia* sp. support our finding that a localized deletion may have drastic effects on growth of the organism. In particular, the possible role of the transporter and its transported substrate in EPS formation deserves further experimental investigation. In addition, the recent findings in *Salmonella* support the assumption that the YiaMNO transporter functions under limiting substrate conditions, where L-xylulose is an intermediate in eukaryotic metabolism. The co-localization of carbohydrate transport and metabolism functions is found in a range of pathogenic bacteria (Plantinga et al., 2004b), suggesting these systems may play important roles in survival during infection. Further studies are required to establish the physiological role of the binding-protein-dependent secondary transporter encoded by the *E. coli* K-12 yiaMNO genes.

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