

The *Escherichia coli* BarA-UvrY Two-Component System Is Needed for Efficient Switching between Glycolytic and Gluconeogenic Carbon Sources

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The *Escherichia coli* BarA and UvrY proteins were recently demonstrated to constitute a novel two-component system, although its function has remained largely elusive. Here we show that mutations in the sensor kinase gene, *barA*, or the response regulator gene, *uvrY*, in uropathogenic *E. coli* drastically affect survival in long-term competition cultures. Using media with gluconeogenic carbon sources, the mutants have a clear growth advantage when competing with the wild type, but using media with carbon sources feeding into the glycolysis leads to a clear growth advantage for the wild type. Results from competitions with mutants in the carbon storage regulation system, CsrA/B, known to be a master switch between glycolysis and gluconeogenesis, led us to propose that the BarA-UvrY two-component system controls the Csr system. Taking these results together, we propose the BarA-UvrY two-component system is crucial for efficient adaptation between different metabolic pathways, an essential function for adaptation to a new environment.

A bacterium has to be prepared for rapid changes in its environment, for example a varying supply of nutrients. The rapid adjustment to new conditions largely relies on two-component systems (TCSs) (20), typically consisting of a membrane-bound sensor protein communicating with a regulatory protein inside the bacterium via phosphotransfer reactions. The importance of TCSs for adaptation during the different steps of infection have consequently made them attractive targets for novel types of antimicrobial therapy (5).

In the prototypic model an environmental stimulus interacts with the N terminus of the membrane-anchored sensor protein, leading to autophosphorylation at a specific histidine residue. The sensor then acts as a kinase and transduces the phosphate group to an aspartate residue on the response regulator protein. This normally enables the regulator to control the transcription of a certain set of genes by sequence-specific DNA binding. Sequencing of the *E. coli* genome has identified approximately 60 sensor kinases and response regulators, most of which have been arranged in cognate pairs (25). In some cases the specific stimulus of the sensor as well as a set of genes under the control of the TCS has also been identified (14, 20).

Hybrid sensor proteins have a more complex architecture and function. Following the initial autophosphorylation of the histidine residue, the phosphate group may be transferred to a second histidine residue in another domain (HPT) of the sensor protein via an aspartate residue, before being relayed to the

regulator protein. The reason for such hybrid sensors is unclear, although they may allow for additional modulation of the signal transduction to the regulator protein or for the ability to regulate different pathways (10, 15, 24).

The hybrid sensor BarA (also called AirS) was first identified by its ability to activate the OmpR response regulator (28). As this effect could only be observed with high-copy-number expression of BarA, it was not believed to be a physiologically relevant function (28). We have recently reported biochemical and genetic evidence demonstrating that the previously orphan BarA sensor protein forms a TCS with the UvrY response regulator protein (30). UvrY resembles a typical response regulator protein of the FixJ family but had no assigned function in the cell. It derives its name from the close linkage on a bicistronic mRNA with the *uvrC* gene, although mutations in *uvrY* have no effect on the UV-light-induced DNA repair system (26). Other work has suggested that one target gene downstream of BarA is the alternative sigma factor *rpoS*, but the physiological context and the nature of the external stimuli still remain elusive (27). TCSs orthologous to BarA-UvrY in other pathogenic gram-negative bacteria, such as ExpS-ExpA in *Erwinia* spp. and GacS-GacA in *Pseudomonas* spp., have a clear link to genes encoding secreted products which are involved in the virulence of the bacteria (19, 30). Moreover, mutations in the *uvrY* homologues *varA* (*Vibrio* spp.) and *sirA* (*Salmonella* spp.) lead to decreased virulence in an infant mouse and a bovine intestinal infection model, respectively (2, 41). This fact and the previously suggested coupling between BarA and iron availability during urinary tract infections (42) encouraged us to analyze the phenotypes of mutations in the Bar TCS in uropathogenic *Escherichia coli*.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
J96	Uropathogenic <i>E. coli</i>	21
DS17	Uropathogenic <i>E. coli</i>	38
AKP013	J96 <i>barA</i> ::Kan ^r	This study
AKP159	J96 <i>uvrY</i> ::Kan ^r	This study
AKP221	DS17 <i>barA</i> ::Kan ^r	This study
AKP168	DS17 <i>uvrY</i> ::Kan ^r	This study
MC4100	Str ^r	American Type Culture Collection
AKP014	MC4100 <i>barA</i> ::Kan ^r	30
AKP023	MC4100 <i>uvrY</i> ::Cam ^r	30
MG1655		Michael Cashel
RG1-B MG1655	<i>csrB</i> ::Cam ^r	17
AKP199	MG1655 <i>barA</i> ::Kan ^r (from AKP014)	This study
AKP200	MG1655 <i>uvrY</i> ::Cam ^r (from AKP023)	This study
AKP 287	MG1655 <i>barA</i> ::Kan ^r <i>csrB</i> ::Cam ^r (from RG1-B MG1655)	This study
XL-1 Blue	Tet ^r	Stratagene
Plasmids		
pCA9505	Carries <i>uvrY</i> gene, Amp ^r	26
pCA9505-MluI	Encodes truncated UvrY protein, Amp ^r	This study
pGEM-T easy	T-cloning vector, Amp ^r	Promega
pUC4K	Contains kanamycin resistance cassette	Amersham Pharmacia Biotech
pKO3	Plasmid used in allelic replacement	23
pCVD422	Plasmid used in allelic replacement	11

MATERIALS AND METHODS

Strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. Using P1-*vir* lysates from AKP014 and AKP023, the *barA* and *uvrY* knockout mutations were transduced into MG1655 to create AKP199 and AKP200, respectively. The *barA* knockout mutation in J96 was constructed by allelic gene replacement using integration vector pCVD442-*barA*::Kan^r as previously described (30). To create a *barA* knockout in DS17 we needed to amplify the *barA* gene from DS17 by PCR using primers 5'-GCATACGCCAAAATGAGGACAG-3' and 5'-GAAACCAGCGTCATAAAAAGCC-3'. The 3,119-bp fragment was cloned into the pGEM-T easy vector. A kanamycin resistance cassette was excised from pUC4K with *Bam*HI and ligated into the *Bgl*II site in the *barA* open reading frame (ORF), creating pGEM-T-*barA*::Kan^r. This construct was digested with *Not*I to release the *barA*::Kan^r fragment, which was subsequently subcloned into pKO3 between the *Not*I sites. The resultant construct was transformed into XL-1 Blue cells that were grown at 30°C on kanamycin (50 µg/ml) plates. Plasmids were purified and retransformed into DS17. The pKO3-*barA*::Kan^r cointegrates were selected at 44°C on Luria broth (LB) plates containing kanamycin. Resolution products were subsequently selected for growth on 5% sucrose at 30°C. To construct the *uvrY* knockouts in J96 and DS17 we first amplified the chromosomal genes from these strains by PCR using primers 5'-CGAAATATTCACCTTTGGC-3' and 5'-GGGAAAAGGACAAAAGC-3' (for J96) and primers 5'-CACCACCTCAGGAAGATAAAAAG-3' and 5'-GGGAAAAGGACAAAAGC-3' (for DS17), creating 2,063- and 2,124-bp products, respectively. The PCR fragments were cloned into the pGEM-T easy vector, and a kanamycin resistance cassette, excised from pUC4K with *Eco*RI and blunt ended using a Klenow fragment, was ligated into the unique *Hinc*II site in the *uvrY* ORF, creating pGEM-T-*DS17uvrY*::Kan^r and pGEM-T-*J96uvrY*::Kan^r. The *uvrY* genes were excised with *Not*I and subcloned into pKO3 between its *Not*I sites. The resultant constructs were used for allelic replacement as described above. All knockouts were confirmed by PCR. The mutant version pCA9505-*Mlu*I was created by opening the unique *Mlu*I site in the *uvrY* ORF and blunting the ends with a Klenow fragment before religation and confirmation of the resultant construction by DNA sequencing.

Growth of cultures. Cultures were grown in LB (10 g of Bacto Tryptone, 5 g of Bacto yeast extract, 5 g of NaCl per liter), in Kornberg medium with a pH of 6.8 (1.1% [wt/vol] K₂HPO₄, 0.85% [wt/vol] KH₂PO₄, 0.6% [wt/vol] yeast extract containing 0.5% [wt/vol] glucose per liter), in Kornberg medium in which the phosphate salts had been replaced with 0.2 M 3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxypropanesulfonic acid (AMPSO) adjusted with KOH to a pH of 9.0, or in M9 minimal medium (8.5 g of Na₂HPO₄ · 2H₂O, 3 g of KH₂PO₄, 0.5 g of NaCl, 1 g of NH₄Cl, 1 mM CaCl₂, 1 mM MgSO₄, and 1 µg of vitamin B₁ per liter) with carbon sources added as indicated in the described experiments, using

100-ml Erlenmeyer glass flasks at 37°C in a shaker at 200 rpm. In competition experiments individual cultures were inoculated 1:100 from overnight cultures, directly started from samples in vials at -80°C, and grown to an optical density (*A*₅₉₀) of 0.30 before mixing in a new flask, leading to a total volume of 20 ml. In the recompetition experiments we isolated individual colonies from the 10-day-old cultures and performed a new competition with the original counterpart strain. No antibiotics were included except for the plasmid competition experiments, in which ampicillin was added to 100 µg/ml.

To estimate the amount of CFU, samples of 100 µl were withdrawn from the cultures at the indicated time intervals, subjected to appropriate dilution in phosphate-buffered saline, and plated on LB plates using glass beads. To determine the relative ratio of mutant versus wild type, two approaches were used, both of which yielded similar results. Either approximately 100 individual colonies from the LB plates were restreaked onto selective plates or the dilutions were plated on both LB plates without antibiotics and LB plates containing the appropriate antibiotic.

CAS assays. Chrome Azurol S (CAS) agar plates were made using the protocol of Schwyn and Neilands (34) with modifications according to Fiss and Brooks (13), which can be summarized as follows: 700 ml of basal CAS medium [0.3 g of KH₂PO₄, 0.5 g of NaCl, 1.0 g of NH₄Cl, 30.2 g of piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES) (pH 6.8), 5 g of L-asparagine, and 15 g of agar per liter] was autoclaved, and 280 ml of the plate CAS indicator solution (34) was added slowly, followed by the addition of 20 ml of sterile glycerol. Bacteria were grown overnight in LB at 37°C with shaking and were reinoculated 1:100. At an optical density (*A*₅₉₀) of 0.15, the iron chelator desferrioxamine was added to a final concentration of 500 µM. The cultures were grown until stationary phase, and samples of 100 µl were withdrawn, subjected to appropriate dilutions in phosphate-buffered saline, and plated onto CAS-containing agar plates that were incubated at 30°C for 2 days. The yellow zone around the colonies, indicating siderophore production, was measured.

The fluid CAS assay was conducted according to the method of Schwyn and Neilands (34) with minor modifications. The fluid CAS indicator shuttle solution was made by adding a mixture of 1.5 ml of 1 mM FeCl₃ · 6H₂O in 10 mM HCl and 7.5 ml of 2 mM CAS with vigorous stirring into a 100-ml flask containing 6 ml of 10 mM hexadecyltrimethyl ammonium bromide. In a 50-ml Falcon tube, 2.1 g of anhydrous piperazine was dissolved in 10 ml of distilled water, into which approximately 3.1 ml of 12 M HCl was slowly added to obtain a pH of 5.6, and this was added to the above-mentioned 100-ml flask. The final volume was adjusted to 100 ml using distilled water, and finally 0.1 g of 5-sulfosalicylic acid was added. Bacteria were grown overnight at 37°C with shaking in M9 minimal medium with 0.2% (wt/vol) glucose and were reinoculated 1:100. The optical density (*A*₅₉₀) was monitored, and 500-µl aliquots were withdrawn at the indi-

cated time point and centrifuged at $220 \times g$ for 5 min. Equal volumes of culture supernatant and fresh CAS indicator shuttle solution were mixed in 96-well plates and left for 60 min at room temperature with shaking. The negative control consisted of a parallel tube with no bacteria inoculated. Siderophore production was assayed by measuring A_{620} in an iEMS reader (Labsystems OY, Vantaa, Finland). The A_{620} shown in graphs has been calculated as follows: $A_{620}(\text{CAS indicator shuttle solution and M9 minimal medium}) - A_{620}(\text{CAS indicator shuttle solution and the bacterial supernatant})$.

RESULTS

Individual cultures of mutants in the Bar TCS exhibit no apparent difference in growth kinetics. Searching for a phenotype of an impaired BarA-UvrY TCS, we first constructed inactivating mutations in the *barA* and *uvrY* genes in the uropathogenic J96 and DS17 *E. coli* strains. The growth of the *barA* and *uvrY* mutant strains was first tested individually under a series of standard conditions. Neither mutant exhibited a measurable difference in growth compared with the wild type when growth was tested on a wide range of different media, as measured by optical density and CFU. The tested strains also reached the same density and showed no difference in the length of the lag phase to resume growth when reinoculated into fresh medium (data not shown).

Mutants in the Bar TCS take over in a competition with the wild type in LB. Some defects in fitness might not be detected by monitoring the growth of single cultures. A more discriminatory method is competition between mutant and wild-type strains during extended growth of batch cultures. The mutant (*barA* or *uvrY*) and isogenic wild-type J96 or DS17 strains were grown individually into the mid-exponential phase in LB, and then equal numbers of cells were mixed in an aerated glass flask and left to shake at 37°C for several days (in triplicate). Samples were collected at different time intervals to quantify the number of wild-type and mutant cells. Under this condition, the *barA* mutant, as well as the *uvrY* mutant, outcompeted the wild-type strain. With the J96 strain it took approximately 4 days from the start of the competition for either mutant to predominate in the culture ($>90\%$ of total bacteria), but with the DS17 strain we could observe this takeover already on the second day (Fig. 1a to d). It should however be noted that individual cultures of mutant (*barA* or *uvrY*) and wild-type J96 or DS17 strains all exhibited similar long-term survival patterns, as demonstrated by equal numbers of CFU, when they were grown in LB for 10 days (Fig. 1e).

If the mutants have a true fitness gain in a competition they should be able to take over the culture when inoculated as a minority. This was demonstrated by the observation that both the J96 and DS17 *barA* and *uvrY* mutants could completely take over their respective cultures after inoculation in a 1:9 ratio relative to the wild type (Fig. 2a).

The phenotype is reversed by complementation with a *uvrY* gene in trans. To exclude an effect of the resistance marker genes, used to create the knockouts of the *barA* and *uvrY* genes, or of inadvertent secondary mutations, we decided to perform a competition in which we expressed the *uvrY* gene in trans from a vector. A *uvrY* mutant and a wild-type J96 strain, both containing a plasmid with a functional *uvrY* gene, performed equally well in our competition experiments. However, when the experiment was repeated with both strains containing a plasmid carrying a *uvrY* gene with an inactivating frameshift

mutation, again the *uvrY* mutant strain outcompeted the wild-type strain (Fig. 2b). The J96 strain was adequate in this experiment, as we needed to select for plasmids, but considering the more pronounced takeover we decided to focus our further experiments on the DS17 strain.

The takeover of the mutants coincides with the lysis of the primary cultures. The observed difference in the ratios of wild type and mutant in the competition experiments may derive from different abilities to use the prevalent food source but may also reflect different rates by which different bacteria undergo lysis after entry into stationary phase. By monitoring the growth kinetics and the CFU of individual cultures of the DS17 wild type and its *barA* and *uvrY* mutant derivatives during the first 24 h, we did not observe any significant difference in the killing-off profiles (Fig. 3a). In the competition experiments seen in Fig. 1, it seems as if the mutants, but not the wild type, increase in numbers following the initial killing off. The killing off is a result of massive lysis which provides a good source of carbon for the survivors, mostly in the form of free amino acids (43). A more detailed analysis of the first 24 h of our competition experiment also showed that the mutants start to dominate the cultures around 20 h after mixing, at a point of time when the killing off of the cultures has just started (Fig. 3b), suggesting that the mutants are better at metabolizing this carbon source. We next performed a competition experiment using two individual DS17 cultures, one wild type and the other a *uvrY* mutant, both of which had been grown well into stationary phase for 2 days before mixing them in equal volumes. The *uvrY* mutant could still clearly take over the cultures, supporting the hypothesis that the advantage of the mutants lies in being better able to metabolize the existing carbon source of the exhausted cultures (i.e., amino acids). It should also be pointed out that the wild type and mutant were in a 1:1 ratio at the start of this competition experiment, again showing that there is no difference in the killing-off profiles of the wild type and mutant (Fig. 3c).

The takeover depends on the carbon source in the medium. LB is based on a mixture of protein hydrolysates, wherein small peptides and amino acids are the principal energy and carbon source. Competition experiments between the DS17 wild type and the *uvrY* mutant in minimal media supplemented with specific protein hydrolysates (tryptone or Casamino Acids) or with yeast extract also led to a clear growth advantage of the *uvrY* mutant (Fig. 4a). However, when we extended the same competition experiments to a minimal medium supplemented with glucose as the sole carbon source, it was the wild type that gradually came to dominate the mixed culture (Fig. 4b). Nevertheless, this occurred after approximately 1 week in mixed culture and was thus distinct from the takeover that occurred in LB or protein hydrolysates. Using fumarate, acetate, or pyruvate as carbon sources in the minimal medium, all of which are downstream intermediates of the energy metabolism, we again observed a growth advantage of the *uvrY* mutant, whereas the wild type took over when we were using glycerol, a substrate which feeds into the glycolysis at an early stage (Fig. 4c). Whereas the takeover is very clear in the experiments with acetate and pyruvate, we consistently observed an initial drop in the relative number of mutants in the fumarate experiment before they later took over the culture. To show that this pattern is not due to the occurrence of sponta-

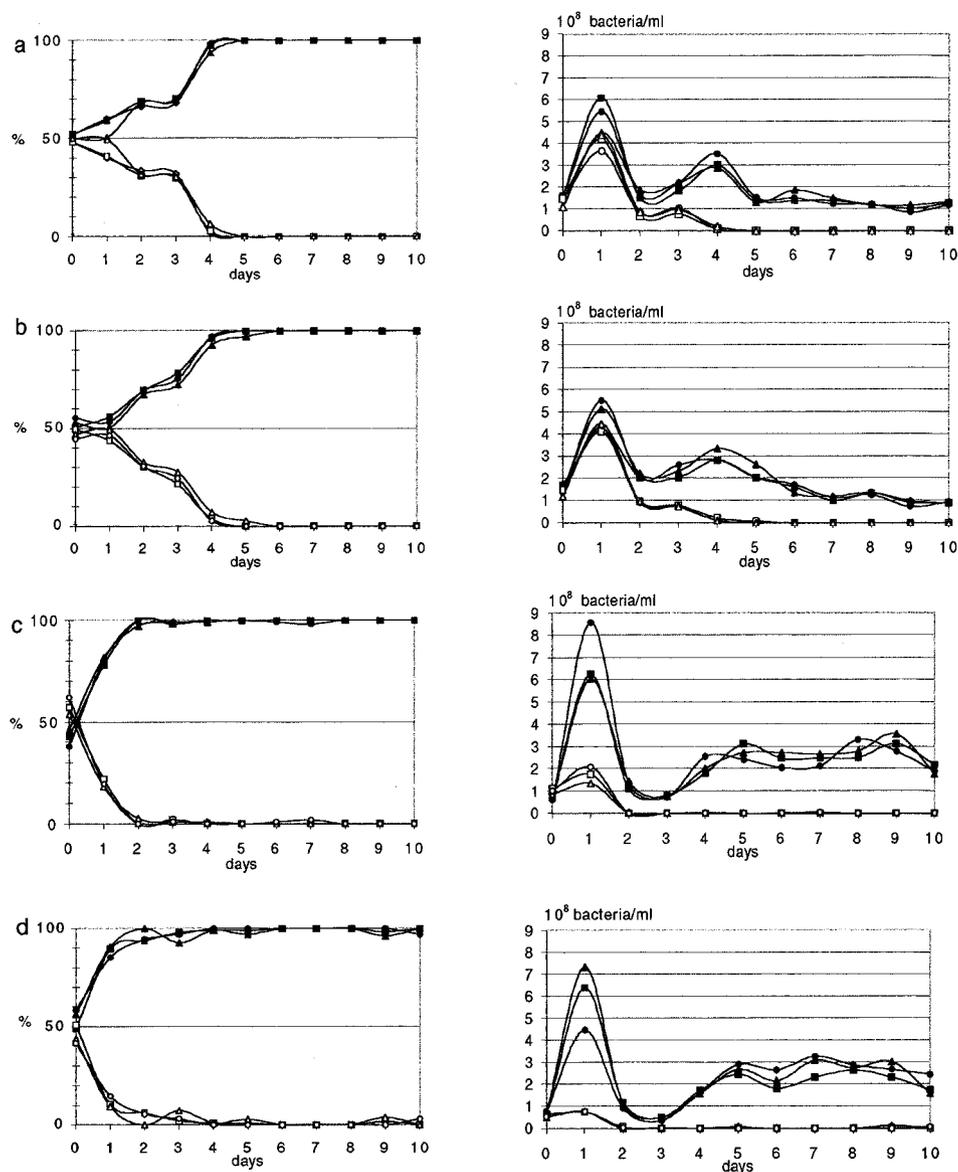


FIG. 1. Mutants take over in long-term competition cultures in LB. Exponentially growing individual cultures in LB of the mutant and its isogenic wild type were mixed 1:1 and split into three flasks, and the resultant cultures were tested for a period of several days for the relative percentages of mutant and wild-type cells. (a) J96 wild type versus J96 (*barA*); (b) J96 wild type versus J96 (*uvrY*); (c) DS17 wild type versus DS17 (*barA*); (d) DS17 wild type versus DS17 (*uvrY*). Graphs to the left show the relative percentage of mutant (●, ■, and ▲) and wild type (○, □, and △) in the mixed cultures. Graphs to the right show CFU (10^8 bacteria/ml) from the same experiments. Other experiments showed that mutants maintain their dominance during longer cultivation periods [30 days with DS17 wild type versus DS17 (*uvrY*)]. (e) Individual cultures of wild type and mutants have similar long-term survival patterns. Graphs show CFU (10^8 bacteria/ml) from individual cultures grown in triplicate in LB for 10 days. To the left DS17 wild type (■), DS17 (*barA*) (●), and DS17 (*uvrY*) (□) are shown, and to the right J96 wild type (■), J96 (*barA*) (●), and J96 (*uvrY*) (□) are shown. Note that cultures already had surpassed their maximal density when the day 1 samples were taken (Fig. 3).

neous mutations, a mutant and a wild type isolated after 10 days of competition were used in different recompetition experiments (against the respective original counterparts) that showed the same takeover pattern as in Fig. 4c (data not shown).

In conclusion however, it seemed that glycolytic substrates led to a growth advantage for the wild type, whereas cultures with carbon sources entering downstream of the glycolysis led to a growth advantage for the *uvrY* mutant in the competition assays. One should be reminded these effects may only be

clearly demonstrated when there is a competition between two strains for the carbon source.

Role of pH in the outcome of competition. We also monitored the pH of competition cultures growing in different media, to see if there was a correlation with the ability of the wild type and the mutants to compete. The pH of the acetate, fumarate and pyruvate cultures moderately increased from being neutral to a slightly basic value, whereas the pH of the glycerol and glucose cultures dropped to an acidic pH (Table 2), thus suggesting that the observed effects could be linked to

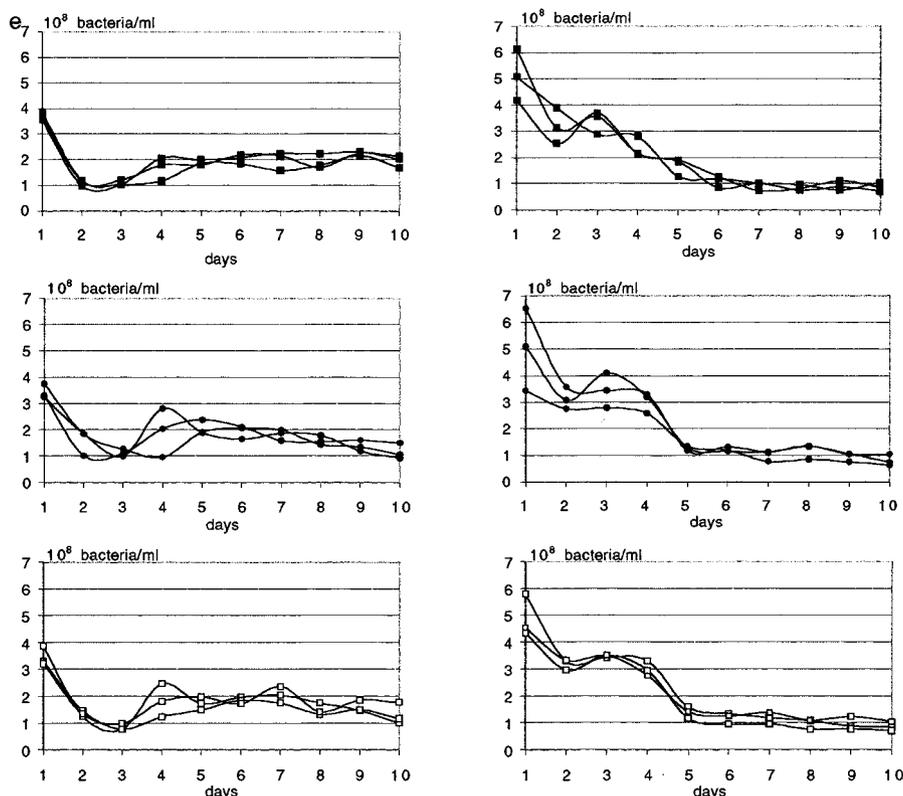


FIG. 1—Continued.

the pH. If the pH value were the single determinant for the outcome of the competition, we would expect the identical takeover profile in different carbon sources at any given pH. Using a phosphate-buffered yeast extract medium (Kornberg medium) we could maintain the same neutral pH with or without glucose in the medium (Table 2). Still the wild type could take over in the glucose culture during the first 4 days, but this could not be seen in the competition without glucose (Fig. 4d). We also repeated this experiment in the same medium buffered to a basic pH using the AMPSO buffer. Here the wild type could never clearly take over the glucose culture,

although the takeover of the mutant was clearly delayed compared with the nonglucose culture (Fig. 4e), demonstrating that pH does have an effect but is not the single factor determining the outcome of the competition.

The Bar TCS regulates the carbon storage regulatory system. The results suggested to us that the BarA-UvrY TCS controls some regulatory system deciding the flow of carbon in the cell. The observed phenotype was reminiscent of that conferred by mutants in the carbon storage regulatory (Csr) system. In this system the CsrA protein acts posttranscriptionally to balance the expression of genes driving glycolysis and glu-

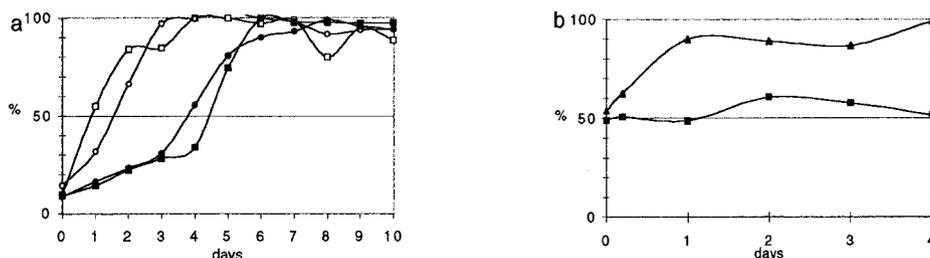


FIG. 2. (a) Mutants take over when inoculated as a minority. Exponentially growing individual cultures in LB of the mutant and its isogenic wild-type parent strain were mixed in a 1:9 ratio in four different competition cultures. The resultant cultures were tested for a period of several days for the relative percentage of mutant and wild type. Graphs show only the percentage of the mutants from each competition mixture: J96 ($barA$) (\blacksquare) J96 ($uvrY$) (\bullet), DS17 ($barA$) (\square), and DS17 ($uvrY$) (\circ). (b) The growth advantage is directly linked to the BarA-UvrY TCS. Exponentially growing individual cultures in LB of J96 wild type and J96 ($uvrY$) were mixed 1:1 and competed in two separate experiments over a period of several days. In the first experiment both competing strains harbored a plasmid carrying a wild-type $uvrY$ gene [J96 ($uvrY$)/pUvrY versus J96 wild type/pUvrY] (\blacksquare), and in the second experiment both competing strains carried the same plasmid with a mutated $uvrY$ gene [J96 ($uvrY$)/pUvrY-mut versus J96 wild type/pUvrY-mut] (\blacktriangle). Only the relative percentage for the plasmid-containing J96 ($uvrY$) is shown for each experiment.

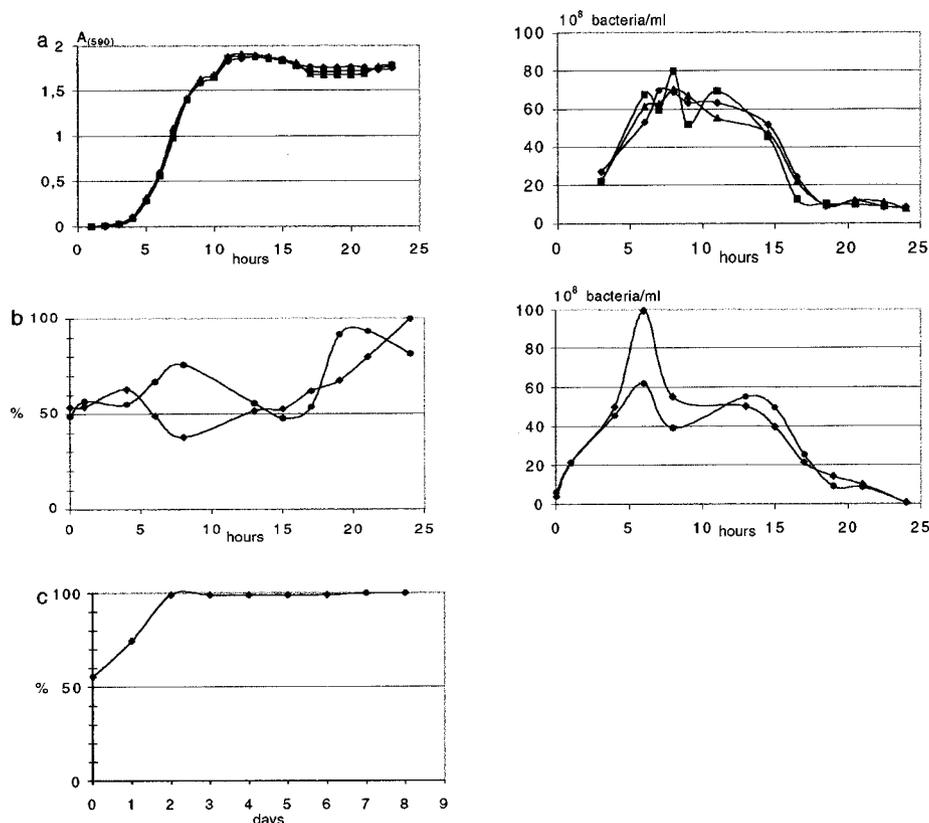


FIG. 3. (a) Wild type and mutants have the same killing-off profiles. DS17 wild type (\blacklozenge), DS17 (*barA*) (\blacktriangle), and DS17 (*uvrY*) (\blacksquare) were grown as individual cultures in LB for 24 h. The density of the bacteria was monitored by A_{590} (left). CFU from the same experiments (right) are shown for DS17 wild type (\blacklozenge), DS17 (*barA*) (\blacktriangle), and DS17 (*uvrY*) (\blacksquare). (b) The takeover of the mutants coincides with the lysis of the primary cultures. Exponentially growing individual cultures of mutants and wild type in LB were mixed at an A_{590} of 0.3 in a 1:1 ratio, and the resultant cultures were tested over a period of 24 h for the relative percentages of mutant and wild type. The graphs show the relative percentages (left) of DS17 wild type versus DS17 (*barA*) (\bullet) and DS17 wild type versus DS17 (*uvrY*) (\blacklozenge). Total CFU (10^8 bacteria/ml) (right) from the same competition mixtures. (c) The mutant takes over in a competition between two stationary-phase cultures. DS17 wild type and DS17 (*uvrY*) were grown for 2 days as individual cultures in LB and then mixed in equal volumes, after which the relative percentage was determined in the mixed culture for a period of several days. The graph shows the percentage of DS17 (*uvrY*) in the mixed culture.

conogenesis, respectively. The activity of the CsrA protein is counteracted by the untranslated CsrB RNA, which can bind up to 18 copies of the CsrA protein (32). If our competition results were to be explained by the Bar TCS acting on the Csr system (35), we were interested to see how a *csrB* deletion mutant, as well as a *csrB-barA* double mutant, behaved in competition assays with a wild type. Experiments were done in the domesticated K-12 strain MG1655, in which we had already made a *csrB* mutation. We first showed that *barA* (and *uvrY*) mutants of MG1655 exhibited a similar takeover pattern in LB when competing against the wild type as did the mutated isolates of DS17 and J96 strains (Fig. 5a and b). In the competitions between MG1655 wild type and the *csrB* (or the *csrB-barA*) mutant in LB, the mutants clearly outcompeted the wild type after a period of approximately 5 days (Fig. 5c and d), which is expected if CsrB is a part of the same signal transduction chain as BarA and UvrY. Moreover, in a competition between the *barA* and the *csrB* mutants in LB, the latter could take over the culture, suggesting that deletion of the *csrB* gives a stronger phenotype than deletion of *barA* (Fig. 5e). We could also show that the *csrB* mutant, like the DS17 *uvrY* mutant, loses in a competition against the wild type using a glycolytic

medium (Fig. 5f). The link between BarA-UvrY and CsrB-CsrA is also supported by our recent results showing that expression of the *csrB* gene is lowered, but not abolished, in strains with a disrupted Bar TCS (35). During the initial phase of the competitions, the *csrB*, or the *csrB-barA*, mutants clearly decreased in the relative percentage. The reason for this is unclear, but it may be connected with pleiotropic effects of excess CsrA during the initial exponential growth phase. We did not include the MG1655 *csrA* mutant strain in the competition assays as it had a growth defect and thus was not amenable for this type of analysis.

Iron uptake is not directly controlled by the Bar TCS. It was previously suggested that the limited amount of iron in urine should make the DS17 *barA* mutant less virulent in the urinary tract, since it appeared to be deficient in iron uptake (31, 42). However, our more refined measurements with a fluid CAS assay do not show such a difference. Using the J96 strain there is no difference in the amount of siderophores produced between the wild type and the *barA* (or *uvrY*) mutants (Fig. 6). Our experiments also failed to demonstrate any retardation of growth of the *barA* and *uvrY* mutants in media chelated with desferrioxamine. Lastly, we analyzed the expression of *lacZ*

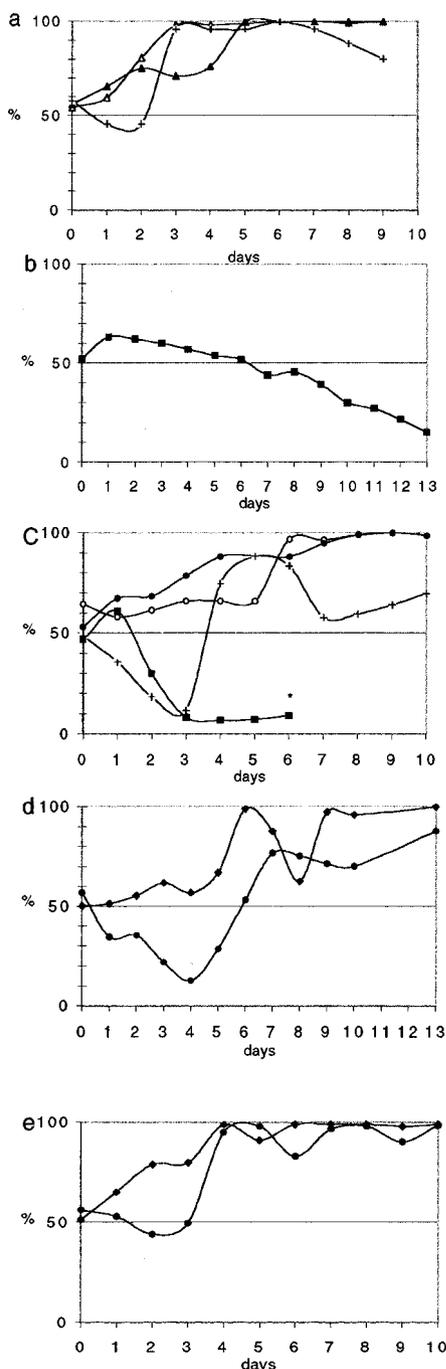


FIG. 4. Competition in minimal medium with different carbon sources and in Kornberg medium. Exponentially growing individual cultures of DS17 (*uvrY*) and DS17 wild type in the indicated media were mixed 1:1, and the resultant culture was tested for a period of several days for the relative percentage of mutant and wild type. (a) M9 minimal medium supplemented with tryptone (24 g/liter) (Δ), Casamino Acids (24 g/liter) (\blacktriangle), or yeast extract (24 g/liter) (+). (b) M9 minimal medium complemented with 0.5% (wt/vol) glucose. (c) M9 minimal medium complemented with 50 mM acetate (\circ), 50 mM pyruvate (\bullet), 50 mM fumarate (+), or 50 mM glycerol (\blacksquare). (d) Kornberg medium buffered with potassium phosphate (pH = 6.8) with 0.5% (wt/vol) glucose (\bullet) and without glucose (\blacklozenge). (e) Kornberg medium buffered with AMPSO (pH = 9.0) with 0.5% (wt/vol) glucose (\bullet) and without glucose (\blacklozenge). Graphs show the relative percentage of DS17 (*uvrY*) in the mixed culture, and the data are from one of several representative experiments. *, culture in glycerol died after day 6.

fusions of the key genes encoding the enterocholin siderophore, *entC* and *entF* (29) (kindly provided by M. McIntosh), without observing any difference in expression between *barA* (or *uvrY*) mutants and the wild type (data not shown).

DISCUSSION

The experiments described herein identify the BarA-UvrY TCS as a determining factor for the ability of *E. coli* to compete during long-term cultivation. This effect is dependent on the carbon source of the growth medium. Using glycolytic substrates, such as glucose, a *uvrY* mutant loses against the wild type in competition experiments. Growth on gluconeogenic substrates, such as amino acids, permits the mutant to dominate the culture. Our experiments also demonstrated that deletion of the *csrB* (carbon storage regulation) gene, which controls the shift between gluconeogenesis and glycolysis, gives a phenotype similar to the one observed with *barA* or *uvrY* mutants.

The Bar TCS regulates the Csr system. From these results we propose a model where the BarA-UvrY TCS regulates the metabolic pathways of carbon metabolism via the CsrA/CsrB regulatory system, by positively controlling the expression of the untranslated CsrB RNA (Fig. 7). It is known that higher levels of CsrB RNA will lead to sequestration of the CsrA protein, an RNA binding protein. The CsrA protein in turn balances the carbon flow in the cell by activating the expression of genes of the glycolysis and by repressing the genes involved in gluconeogenesis, in a posttranscriptional manner (32, 33).

A direct regulatory effect of the BarA-UvrY TCS on CsrB has been demonstrated in a parallel work, where it was shown that a *uvrY* mutant fails to accumulate CsrB RNA and is defective for expression of a *csrB-lacZ* fusion (35). Further evidence for this link may also be found in other bacterial species that have untranslated RNAs analogous to that of CsrB. Although these RNAs are not highly conserved at the nucleotide level, they have a similar structural organization and are thought to bind multiple copies of CsrA-like proteins. Comparison of the annotated database sequences shows that the CsrA protein itself is relatively homologous between these species. In *Pseudomonas*, mutations in the Gac TCS could be suppressed by a CsrA-like protein (7) or a regulatory PrrB RNA (1); the CsrA-like protein and PrrB, like the recently described RsmZ (18), appear to be analogous to CsrB. Recent work with *Erwinia* has also provided genetic evidence that the homologous TCS regulates its target genes via a CsrA/CsrB-like system (9, 22).

In *Salmonella* the *csrA*, *csrB*, *barA*, and *sirA* genes have been reported to be involved in invasion, suggesting a genetic link between these systems (3, 4). The proposed link between BarA-UvrY and the Csr system also agrees well with two recent publications—one showing that several UvrY homologues can control flagellar gene expression (16) and the other showing that the Csr system in *E. coli* has a similar function (40).

The results from our competitions are also influenced by the pH of the culture, as may be inevitable considering the importance of pH for the metabolism of a cell. One crucial factor is that uptake and utilization of different carbon sources vary with the pH, for example glucose utilization proteins are down-regulated at a high pH and up-regulated at a low pH (6), which

TABLE 2. pH in competition cultures^a

Day(s) of growth	pH of culture grown with:									
	50 mM Fumarate	50 mM Acetate	50 mM Pyruvate	50 mM Glycerol	0.5% Glucose	Kornberg K ₂ HPO ₄ ^b		Kornberg AMPSO ^b		
						+Glucose	-Glucose	+Glucose	-Glucose	
0	7.2	7.0	7.0	7.0	6.8	6.8	6.8	9.0	9.0	
1	7.2	7.2	7.2	6.4	5.6	6.8	6.8	8.5	9.0	
2	7.2	7.2	7.2	5.8	5.6	6.8	6.8	9.0	9.0	
3	7.2	7.5	7.2	4.8	5.6	6.8	6.8	9.0	9.0	
4	7.2	8.0	7.2	4.8	5.8	6.8	6.8	9.0	9.0	
5	7.2	8.0	7.2	5.1	5.8	6.8	6.8	9.0	9.0	
6	7.5	8.0	7.5	5.1	5.8	6.8	6.8	9.0	9.0	
7	7.7	8.0	7.5	5.2	5.8	6.8	6.8	9.0	9.0	
8	7.7	8.0	7.7	5.2	5.8	6.8	6.8	9.0	9.0	
9	7.7	8.0	7.5	5.2	5.8	6.8	6.8	9.0	9.0	
10	7.7	8.0	7.5	5.2	5.8	6.8	6.8	9.0	9.0	

^a By using indicator strips, the pH was monitored in competition cultures between DS17 (*uvrY*) and DS17 wild-type strains grown in minimal medium and in two different Kornberg media supplemented with the indicated carbon sources.

^b The pH with 0.5% glucose (+Glucose) and the pH without glucose (-Glucose) are shown.

could explain the inability of the wild type to outcompete the mutant in the glycolytic medium at pH 9. The results do not imply that pH is the sole reason why competitions in different carbon sources give different outcomes. We could always ob-

serve a clear effect of the carbon source itself in our experiments, no matter what the pH was, and we see no evidence that pH acts via the BarA-UvrY system.

Another confounding factor in this type of competition stud-

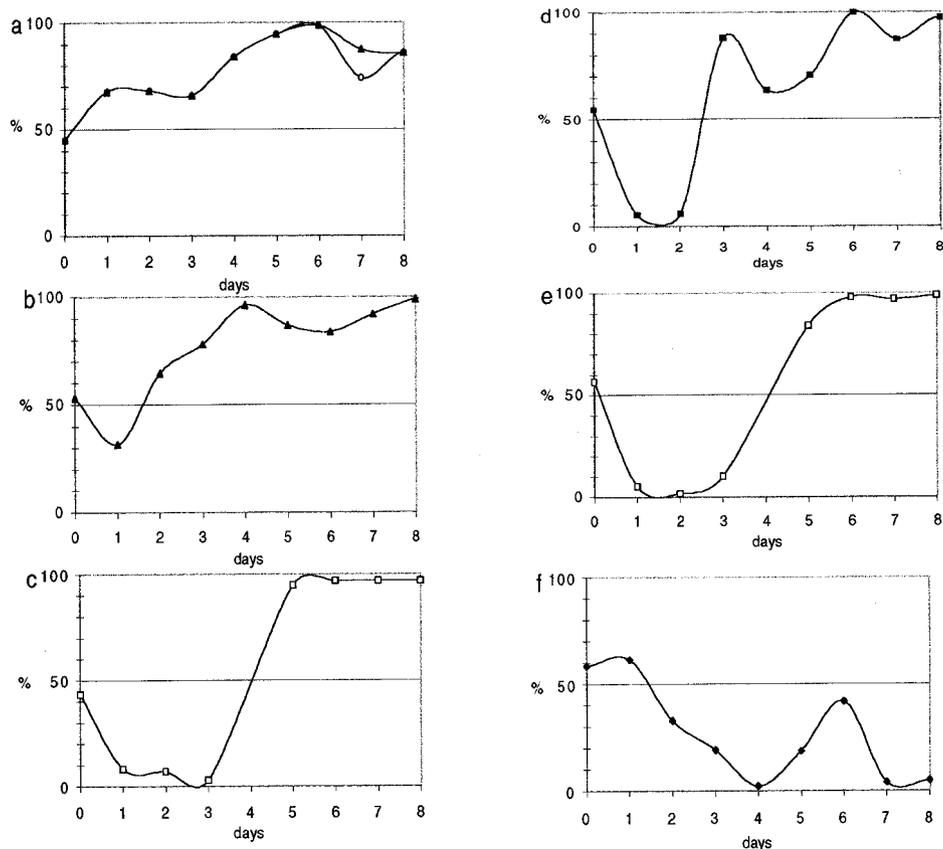


FIG. 5. The Bar TCS regulates the Csr system. (a) *E. coli* MG1655 mutants outcompete the wild type. Exponentially growing individual cultures of MG1655 (*uvrY*) (▲) or MG1655 (*barA*) (○) and MG1655 wild type in LB were mixed 1:1, and the resultant cultures were tested for a period of several days for the relative percentage of mutant and wild type. (b) The takeover is not due to secondary mutations. An isolate of MG1655 (*uvrY*) (▲) from a 10-day competition culture (from panel a) was used in a recompetition with the original MG1655 wild type. (c) The MG1655 (*csrB*) (□) takes over when competed with MG1655 wild type in LB. (d) The MG1655 (*csrB barA*) (■) takes over when competed with MG1655 wild type in LB. (e) The MG1655 (*csrB*) (□) takes over when competed with MG1655 (*barA*) in LB. (f) The MG1655 wild type takes over when competed with MG1655 (*csrB*) (◆) using 0.5% glucose in Kornberg medium.

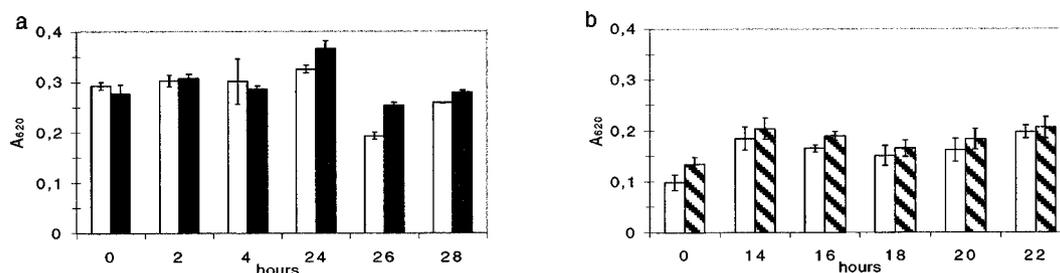


FIG. 6. Siderophore levels are similar in mutant and wild type. Individual cultures of J96 wild type, J96 (*uvrY*), and J96 (*barA*) were grown in minimal media with 0.2% glucose over a period of several days. Samples were harvested at different time points after entry into stationary phase (0 h). The amount of secreted siderophores was measured by the CAS fluid assay in the supernatant as A_{620} . Data are from one of several representative experiments with parallel plates. Error bars indicate standard deviation between the plates in one experiment. (a) J96 wild type (open bars) and J96 (*barA*) (filled bars); (b) J96 wild type (open bars) and J96 (*uvrY*) (striped bars).

ies is the secretion of secondary metabolites into the cultures, which themselves could serve as carbon sources at later stages of the culture. This may, together with the pH, explain the apparently irregular takeover profiles we observed in some of our experiments.

The Bar TCS facilitates switching between different carbon sources. This model implies that a cell without a functional BarA-UvrY two-component system will not have the same flexibility to switch between different metabolic pathways. Even in media containing a single glycolytic carbon source, such as glucose, different metabolic pathways will be employed during the growth of a long-term bacterial batch culture. Initially, glucose will be metabolized via glycolysis. As levels of glucose decrease, the bacterium will start to metabolize products of glycolysis that have accumulated in the medium, such as acetate and alpha-keto acids (36). When these carbon sources have been depleted, metabolism will rely on carbon sources from lysed bacteria, primarily consisting of amino acids. As between 90 and 99% of the bacteria will die soon after entry into the stationary phase, the amino acids will soon constitute a major carbon source for de novo synthesis (43). This model agrees well with our results of the wild type taking over the mixed cultures based on glycolytic carbon sources.

It is less obvious why the *barA* and *uvrY* mutants take over the competition cultures in media with gluconeogenic carbon sources. One reason could be that they are defective in shifting between metabolic pathways, which would not be needed during these long-term cultivations, since energy is provided by carbon sources directly fuelling the Krebs cycle. It has also been demonstrated that under certain conditions CsrA affects amino acid uptake (39). Another reason could be that the relative down-regulation of gluconeogenic enzymes in the mutants, via the Csr system, confers a growth advantage for the mutants, as these pathways would be of less importance and rather be a burden for the wild-type bacteria. Our results are also supported by earlier work showing that mutations conferring an enhanced amino acid catabolism will gain a growth advantage in stationary phase (43) (see below). In *Pseudomonas* the genes encoding the Gac TCS have been observed to be targets for spontaneous mutations that lead to an apparent growth advantage in rich media (19), an effect which is eliminated by the addition of low concentrations of certain divalent ions to the growth medium (12). We have tested similar addi-

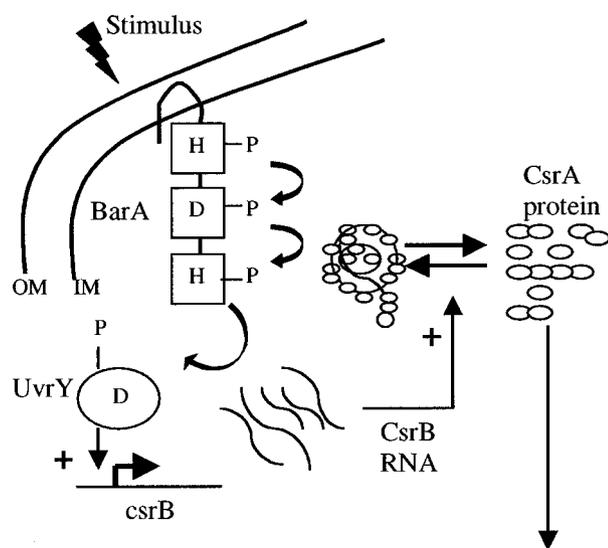
tions of ions without observing any effect on the survival of our mutants (data not shown).

Bar TCS and the GASP phenotype. The original carbon source will eventually become depleted in long-term cultures, and the residual growth will then be based on amino acids derived from lysed bacteria. We would thus expect a takeover of the mutants in all cultures if they were allowed to continue for this length of time. This picture is, however, complicated by the fact that it may take a very long time for the mutant to resume domination of the culture if it has been strongly diminished in numbers during the initial competition. Another confounding factor is the occurrence of spontaneous mutants with a growth advantage in stationary phase (GASPs), which have been described to arise in such extended cultures with a frequency of one new GASP approximately every 7 days (43). It is, however, very unlikely that our results should be explained by such spontaneous mutations in the culture, given that our recompetitions exhibited similar takeover profiles as the original competition. We could also observe a similar takeover in several parallel cultures.

Intriguingly, some of these GASP mutations were mapped to the genes encoding stationary-phase sigma factor RpoS and the leucine-responsive protein (44). Both of these are key regulators of metabolic functions in the cell, and the increased fitness of some mutants was explained by the enhanced ability to catabolize amino acids from lysed bacteria.

Physiological role of Bar TCS. TCSs orthologous to BarA-UvrY in other pathogenic gram-negative bacteria have been shown to be involved in the regulation of virulence traits (19, 30). This fact and the previously suggested coupling between BarA and urinary tract infections (42) suggested to us that the capability of switching between different carbon sources could give the bacteria a higher fitness in urine. Urine contains several possible carbon sources, which may vary substantially between different samples (8). Moreover, during cystitis the bacteria will encounter a continuous supply of fresh urine, implying the importance of being able to sense the existing carbon source. Preliminary results in urine culture, in which we allowed the DS17 *uvrY* mutant and wild type to compete for 8 days, showed that the *uvrY* mutant initially exhibited a slight growth advantage, but after a few days the wild type predominated in the culture.

We previously observed an apparent increase in the sid-



Metabolic step	Genes	Response (+, -, 0)
Glycogen synthesis	<i>glgC</i>	-
	<i>glgA</i>	-
	<i>glgB</i>	-
	<i>glgS</i>	-
Glycogen catabolism	<i>glgP</i>	-
	<i>glgX</i>	0
Gluconeogenesis	<i>pckA</i>	-
	<i>fbp</i>	-
	<i>pps</i>	-
	<i>pgm</i>	-
Glycolysis	<i>pgi</i>	+
	<i>pfkA</i>	+
	<i>pfkB</i>	-
	<i>tpi</i>	+
	<i>eno</i>	+
	<i>pykF</i>	+
	<i>pykA</i>	0
Pentose phosphate shunt	<i>zwf</i>	0
	<i>gnd</i>	0
Glyoxalate shunt	<i>aceB</i>	+
	<i>aceA</i>	+
Acetate metabolism	<i>acs</i>	+
TCA-cycle	<i>icd</i>	0
	<i>gltA</i>	0

FIG. 7. Model for regulation of carbon metabolism via the BarA-UvrY TCS. Stimulation of the inner-membrane-bound tripartite BarA histidine sensor kinase by an unknown stimulus leads to phosphorylation of the cognate UvrY response regulator. The phosphorylated UvrY regulator acts positively on the transcription of the *csrB* gene. Increased levels of the untranslated *csrB* RNA lead to sequestration of free CsrA protein molecules and force the equilibrium between bound and nonbound CsrA to the left. In a typical cell it has been estimated that 16 to 32% of the CsrA protein is bound to *csrB* (17). Free CsrA protein has the indicated effects on the carbon metabolism by posttranscriptional regulation (32). Our results imply the importance of this TCS for growth in media where the bacteria could benefit from rapid switches between different types of carbon sources. CsrA has been shown to activate (+), to repress (-), or not to affect (0) the indicated genes. Abbreviations: OM, outer membrane; IM, inner membrane.

erophore levels on a plate CAS assay in the presence of over-expressed UvrY regulator protein in the K-12 strain MC4100 (31), but in the present work, using the fluid CAS assay, we could not detect such a difference. Our previous result (31) could be explained by the Bar TCS acting on the Csr system. The availability of the siderophore precursor chorismate may differ in these assays, supported by a recent study showing that CsrA controls central carbon flux into the aromatic pathway (37). It is also important to note that carbon sources and growth modes differ between the plate CAS assay (glycerol) and the fluid CAS assay (minimal medium with glucose).

Stimulus of the Bar TCS. The physiological stimulus of the sensor has so far not been identified for BarA or any of the orthologous TCSs. It has been suggested that one common denominator is the sensing of the host organism by the bacteria during an infection, as many of the target genes encode secreted proteins involved in the pathogenesis (19, 30). Our results, however, show this system is active in the absence of cell attachment and in the absence of any host organism. From our data, we would prefer to speculate that the BarA sensor could monitor some metabolic product that is representative of the prevalent energy status in the medium. The ability to determine the available carbon sources combined with a rapid modulation of the major energy-generating metabolic pathways certainly would be a key factor for a successful bacterial adaptation and survival. However, this does not exclude attachment to a host cell as a triggering event, since attachment could well create a microenvironment with an increased concentration of the stimulus in a quorum-sensing mode.

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