Transcription of arcA and rpoS during growth of Salmonella typhimurium under aerobic and microaerobic conditions

Mojmír Ševčík, Alena Šebková, Jiří Volf and Ivan Rychlík

Author for correspondence: Ivan Rychlík. Tel: +420 5 41321241. Fax: +420 5 41211229. e-mail: rychlik@vri.cz

Veternářské Národní výzkumné centrum, Hudcova 70, 621 32 Brno, Czech Republic

Physiology of the exponential and stationary phase of growth, under both aerobic and microaerobic conditions, of Salmonella typhimurium and its isogenic mutants nuoG::Km, cydA::TnphoA, ΔarcA and ΔrpoS was studied using luxAB transcriptional fusions with the rpoS and arcA genes. In the wild-type strain, rpoS transcription was greater under aerobic than under microaerobic conditions, whereas transcription of arcA was suppressed by aerobiosis. Under aerobic conditions, no interaction between NuoG, CydA, ArcA and RpoS was detected. Under microaerobic conditions, rpoS was suppressed in the nuoG mutant as compared with the wild-type strain, but it was overexpressed in the cydA and arcA mutants. A deletion in the rpoS gene, on the other hand, resulted in non-restricted, increased arcA expression in stationary-phase cultures under microaerobic conditions. Based on the rpoS transcription in the nuoG mutant the authors propose that the decrease in the NADH:NAD+ ratio that occurs when carbon sources become limiting serves as a signal for increased rpoS transcription, while active respiration catalysed by CydA and controlled by ArcA downregulates rpoS transcription. When, finally, the RpoS-controlled stationary phase of growth is reached, arcA is suppressed in an RpoS-dependent fashion. Transition into stationary phase under microaerobic conditions is thus controlled by coordinated action of the RpoS and ArcA regulators, depending on subtle changes in the environment.

Keywords: rpoS/arcA, stationary phase of growth, starvation, luxAB transcriptional fusion

INTRODUCTION

The stationary phase of bacterial growth is most frequently defined as the situation when bacterial multiplication ceases and cell density no longer increases. The stationary phase used to be understood as a simple stage of bacterial population development; however, recent findings have shown that the transition into and survival during stationary phase is under more complex physiological control in Salmonella and Escherichia coli than previously thought (Siegele & Kolter, 1992; Kolter et al., 1993). In other bacterial species, transition into stationary phase has been shown to be controlled by the production of specific secondary metabolites – autoinducers (Cui et al., 1995; Latifi et al., 1996). Multiple factors can lead to the cessation of growth, so the physiological parameters of different stationary-phase cultures may differ considerably. Most attention has been given to nutrient starvation brought about by limiting carbon, nitrogen or phosphate sources (O’Neal et al., 1994; Seymour et al., 1996). However, it is clear that final electron acceptors, among them oxygen as the most preferred, are equally important determinants of bacterial metabolism. Therefore under anaerobic conditions, when electron acceptors are scarce, the stationary phase of growth may be reached not only due to depleted nutrients but also due to limiting electron acceptors. Salmonella is exposed to such an environment upon infection of a susceptible host, after which it quickly multiplies and reaches a density of about 10^8 c.f.u. per g caecal content. This was the reason why we decided to investigate and compare gene tran-
scriptions in stationary-phase Salmonella typhimurium cultures under aerobic and microaerobic conditions.

Central to the metabolism of S. typhimurium and E. coli in the stationary phase of growth is RpoS, the stationary-phase-specific sigma subunit of RNA polymerase (O’Neal et al., 1994; Talukder et al., 1996). This sigma subunit recognizes the promoters of a variety of genes essential in stationary phase and controls their transcription (Lange & Hengge-Aronis, 1991; Hengge-Aronis et al., 1991; Sitnikov et al., 1998). Consistent with the role of RpoS in stationary-phase metabolic control are results showing that rpoS mutants do not survive prolonged starvation (Fang et al., 1992; O’Neal et al., 1994), although one report surprisingly described that spontaneous mutations in the carboxy terminus of RpoS gave rise to mutants better adapted for stationary-phase survival (Zambrano et al., 1993). Salmonella RpoS mutants were also reported to be attenuated for mice (Fang et al., 1992; Nickerson & Curtiss, 1997); their virulence for chickens is unclear (Allen-Vercoe et al., 1997).

Defects in stationary-phase physiology have been also observed in respiration-deficient mutants. ArcA is a member of a two-component signalling system in S. typhimurium and E. coli (Iuchi & Lin, 1988; Iuchi et al., 1989, 1990a) and mutants in which ArcA is inactivated are unable to survive prolonged starvation (Nystrom et al., 1996). Stationary-phase cultures of other respiration-deficient mutants such as cydA (a protein of the cytochrome d oxidase complex) and nuoG (a protein of the NADH dehydrogenase I complex) are unable to suppress multiplication of the isogenic wild-type strain (Zhang-Barber et al., 1997). cydA mutants were also reported to be defective in exiting the stationary phase (Goldman et al., 1996; Siegele et al., 1996). Mutation in nuoG caused Salmonella to be of reduced virulence for chickens (Zhang-Barber et al., 1998).

As some of the defects of respiration-deficient mutants are similar to the defects observed in the rpoS mutant, we were interested in potential interactions between NuoG, CydA, ArcA and RpoS expression. To examine this, we created rpoS–luxAB and arcA–luxAB transcriptional fusions and transferred these fusions into previously characterized cydA and nuoG mutants (Zhang-Barber et al., 1997), newly constructed in-frame deletion mutants ΔrpoS and ΔnuoG, and a wild-type S. typhimurium strain. This allowed us to demonstrate that control of carbon starvation and control of aerobic/biofilm anaerobiosis are two complementary sites of bacterial response to stress conditions.

**METHODS**

**Bacterial strains and growth conditions.** S. typhimurium F98 NalR (resistant to nalidixic acid) and its isogenic mutants nuoG::Km and cydA::TnphoA have been described previously (Zhang-Barber et al., 1997). Construction of ΔrpoS and ΔarcA strains is described below.

The microaerobic environment used for bacterial propagation was created by using 4 ml volumes of LB broth (Difco) in 8 ml capped plastic tubes incubated statically at 37 °C. To avoid introduction of fresh air during sampling, multiple tubes were inoculated and placed into the incubator. At given time intervals, individual tubes were taken out of the incubator, sampled and assayed. After sampling, opened tubes were discarded. Fully aerobic conditions were obtained by growing the culture in 20 ml volumes of LB broth in 300 ml flasks with strong agitation (250 r.p.m.). The stationary phase of growth was defined as the situation when OD600 values of the culture stopped increasing exponentially.

Cultures were inoculated to obtain initial viable counts of approximately 5 × 10^6 c.f.u. ml⁻¹, which allowed initial exponential growth for more than 15 generations, followed by natural transition into stationary phase. Bacterial growth was monitored as increase in OD600 using a Smart Scan 3000 spectrophotometer (Bio-Rad). Viable bacteria were enumerated by using standard decimal dilutions and plating on appropriate agar plates. In control experiments, the pH and redox potential of nutrient broth of growing cultures were monitored using a combination pH electrode and metal redox electrode (WPA).

As part of the study, the influence of direct addition of NADH (final concentration 1 mM), dinitrophenol (50 µM), vanillyl-nonenamide (50 µM), and dicyclohexylcarbodiimide (50 µM) on rpoS transcription was investigated. All these chemicals were from Sigma.

**Construction of transcriptional luxAB fusions and lumino-nescent control. A pair of primers amplifying the 3' end of the rpoS gene was used (Table 1). The PCR product was cloned into the pNQ705L plasmid vector, which contains promoterless luxAB genes downstream from the plasmid multiple cloning site. After electrot trophy of E. coli 17F (E. coli Pulse, Bio-Rad), the recombinant clone was selected by PCR and verified by DNA sequencing (ABI Prism 310 Genetic Analyser). The plasmid was transferred by conjugation into recipient S. typhimurium F98 NalR. As S. typhimurium does not support the replication of R6K ori pir plasmids (i.e. pNQ705L), the Cm⁻ (chloramphenicol-resistant)/NalR transconjugants were those in which the plasmid had integrated into the host genome by homologous recombination. The proper site of insertion in the S. typhimurium chromosome was confirmed by PCR. The same strategy was used for construction of the arcA–luxAB fusion. Because the full sequence of the S. typhimurium arcA gene was not available at the time of genetic manipulation, the sequence was deduced from the available E. coli sequence aligned with partial S. typhimurium sequences from GenBank and from the Genome Sequencing Center server at Washington University, St Louis (http://genome.wustl.edu/gsc/bacterial/salmonella.shtml).

For lumino-nescent detection, 90 µl of the culture was taken at given time intervals and the level of luminescence corresponding to the level of rpoS (arcA) transcription was monitored. To 90 µl of the culture, 10 µl 1% decanal (Sigma) was injected and light production was immediately integrated for the first 10 s (Olsson et al., 1988). Light production was measured using a Luminoskan microplate-format luminescence reader (Lab-system, Finland) and expressed as luminescence units per OD600 unit (RLU).

**Construction of rpoS and arcA deletions.** Deletion in the rpoS and arcA genes was achieved by cloning the modified PCR product into pDM4 plasmid vector (Milton et al., 1996). The modified PCR product was obtained exactly as described by Ho et al. (1989). The resulting PCR product was purified with a QIAquick Gel Extraction kit (Qiagen), double-digested with XbaI and XhoI (primers contained XbaI or XhoI sequences
The presence of the deleted clone was finally verified by DNA sequencing. RpoS inactivation was also verified by their construction of a deletion in arcA, respectively. Primers rpo1 and rpo4 contained XbaI or XhoI restriction site overhangs (underlined), respectively. Primers rpo2 and rpo3 contained complementary 5′ end overhangs (underlined) followed by an extra ‘A’ (bold) due to the addition of an extra ‘A’ by the Taq polymerase in the first-round PCRs. rpoF1 and rpoF2 (and arcF1 and arcF2) were used in construction of luxAB transcriptional fusions; XbaI and SacI overhangs (underlined) enabled cloning into pNQ705L. The luxAR primer is derived from the sequence of the luxA gene.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′–3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cydAF</td>
<td>CTG GGA TCG TCT GAG TAA AG</td>
</tr>
<tr>
<td>cydAR</td>
<td>CGA GAT CTT TCT TCA TCC TG</td>
</tr>
<tr>
<td>IS50</td>
<td>AGG ACG CTA CTT GTG TAT A</td>
</tr>
<tr>
<td>nuoGF</td>
<td>GAG ATC GAA TCT TAT GAC GC</td>
</tr>
<tr>
<td>nuoGR</td>
<td>GGC GGC AAT CAT AGT AAT AC</td>
</tr>
<tr>
<td>rpo1</td>
<td>GTC AGT CTA GAG GCA AAA AAT CGC TAC TAT G</td>
</tr>
<tr>
<td>rpo2</td>
<td>TAA CAA TGT AAA TCT CAC CAA GGT AAA GC</td>
</tr>
<tr>
<td>rpo3</td>
<td>ACA TCG TTA AAG AGC TGA ACG TAT ACC TGC</td>
</tr>
<tr>
<td>rpo4</td>
<td>CAC GTG TCG AGA ACC TGA ATC TGA CGA ACA C</td>
</tr>
<tr>
<td>arc1</td>
<td>GTC AGT CTA GAC TTA GCC TGT TAT GTC TGT G</td>
</tr>
<tr>
<td>arc2</td>
<td>GGG TTA AAC GGA ATG ATC ACC AGG TGT ATG TC</td>
</tr>
<tr>
<td>arc3</td>
<td>CCG TTT AAC CCA GGC GAA TTT AAC ATC CGT GC</td>
</tr>
<tr>
<td>arc4</td>
<td>CAC GTG TCG AGC CTG CAG GTC GCC GCA GAA G</td>
</tr>
<tr>
<td>rpoF1</td>
<td>GTC AGT CTA GAA GAC ACC ACG CAA GAT G</td>
</tr>
<tr>
<td>rpoF2</td>
<td>GAC GTG AGT CTG TGG CCT TTT TTT GAC AAG</td>
</tr>
<tr>
<td>arcF1</td>
<td>GTC AGT CTA GAC CGG AAT TGA CCA TCC GTC</td>
</tr>
<tr>
<td>arcF2</td>
<td>GAC GTG AGC TCG AAT TAA TCC TGC AGG TCG CC</td>
</tr>
<tr>
<td>luxAR</td>
<td>GTG GCT GAT AAG TGA GAA GG</td>
</tr>
</tbody>
</table>

respectively; see also Table 1), purified again with the QIAquick Gel Extraction kit and ligated into XbaI/XhoI-linearized pDM4. After ligation (T4 DNA ligase, US Biochemicals) the recombinant plasmid was electrotransformed into E. coli 17.1pir and CmR colonies were tested by PCR for the presence of the deleted rpoS gene sequence. Recombinant pDM4ArpoS was conjugated into S. typhimurium F98 NalR. As S. typhimurium does not encode the pir protein, the only CmR/NalR colonies were those in which homologous recombination had occurred. After the second homologous recombination, clones were checked for their sensitivity to chloramphenicol and by PCR for those resulting in the deletion. The deleted clone was finally verified by DNA sequencing.

**Control experiments.** Genotypes of all of the strains before and after any genetic manipulation were checked by PCR. Specific cydA and nuoG primers covering the site of TnphoA or kanamycin gene cassette insertion were designed (Table 1). For the luxAB fusion, the control primer external to the amplified 3′ end of the rpoS (or arcA) gene, together with the primer derived from the luxA gene sequence, were used to verify rpoS–luxAB fusion clones (Table 1). The genotypes of the ΔrpoS and ΔarcA mutants were confirmed by PCR and DNA sequencing. RpoS inactivation was also verified by their inability to ferment trehalose (Hengge-Aronis et al., 1991) in a biochemical typing test (Enterotest, Lachema). ArcA inactivation was tested by the sensitivity of the mutant to toluidine blue (Iuchi et al., 1990a).

All the experiments were repeated at least on three independent occasions. Mean values are shown in the figures; individual values did not differ from the mean by more than 15%.

**RESULTS**

**Verification and growth characteristics of S. typhimurium strains**

Mutations in the nuoG and cydA genes were confirmed by PCR. In the luxAB transcriptional fusions, the start codon of the luxA gene was located 55 bp downstream from the rpoS stop codon, and 35 bp downstream from the arcA stop codon. The fusions were successfully transferred into all the strains as confirmed by PCR.

The in-frame deletion in the rpoS gene consisted of one amino acid exchange, Gly-66 to Tyr-66, in addition to a deletion from Tyr-67 to His-170, inclusive. The deletion was confirmed by PCR and DNA sequencing. The mutant was unable to ferment trehalose (Hengge-Aronis et al., 1991). The arcA in-frame deletion consisted of one
reached was 7 aerobic, an increase in pH was observed; the final pH
around
redox potential was around growth. In microaerobic stationary-phase cultures it reached values around aerobic stationary-phase cultures it reached values 10 mV and gradually decreased during growth. In microaerobic stationary-phase cultures the final redox potential was around −210 mV while in aerobic stationary-phase cultures it reached values around −115 mV. Both the pH and redox potential development therefore clearly documented the differences under the two experimental conditions.

Transcription of rpoS and arcA

rpoS and arcA transcription displayed very similar patterns. Under aerobic conditions their transcription culminated in 10-h-old cultures with OD600 values around 0·6. The cultures reached stationary phase after 15 h, with OD600 values approximately 4·0, and soon after, rpoS and arcA transcription dropped nearly to zero (Fig. 1). As determined by RLU values, under aerobic conditions, the maximal levels of rpoS and arcA transcription were very similar (Fig. 1).

Under microaerobic conditions, maximal levels of rpoS and arcA transcription were observed in cultures 11–12 h old with OD600 values around 0·4, 2 h before the stationary phase of growth was reached. When compared with aerobic cultures, maximal microaerobic rpoS transcription was twofold lower. arcA transcription on the other hand was more than twofold higher. Under microaerobiosis, arcA transcription was fourfold greater than rpoS transcription. After the peak in transcription, a decrease in both rpoS and arcA transcription was observed (Fig. 2); however, ongoing transcription of both the genes could be demonstrated in microaerobic cultures up to 7 d old (not shown).

Influence of nuoG, cydA and arcA mutations on rpoS transcription

arcA suppression by aerobiosis has been observed previously (Iuchi & Lin, 1992) but no data on the relationship between rpoS transcription, availability of oxygen and interactions with the respiratory chain have been reported. We were therefore interested in the extent of modification of rpoS transcription caused by mutations in proteins essential for respiration. An rpoS–luxAB fusion was transferred into the cydA, nuoG and ΔarcA mutants. NuoG is a component of NADH dehydrogenase complex I (Zambrano & Kolter, 1993; Falk-Krzesinski & Wolfe, 1998), an enzyme complex that represents an ‘input site’ of the respiration chain. CydA is a component of the cytochrome d oxidase complex (Cotter et al., 1997) and thus represents a ‘terminal site’ in the respiratory chain. ArcA is a regulatory protein required for the transition from aerobic to microaerobic bacterial metabolism (Iuchi et al., 1990a).

In aerobically grown cultures, rpoS transcription in the nuoG, cydA and ΔarcA mutants and the wild-type strain
Interaction of RpoS and ArcA

Increased rpoS transcription in the ΔarcA mutant indicated that there is an interaction between respiratory control and stationary-phase metabolism controlled by RpoS. Therefore, the influence of rpoS deletion on arcA transcription was determined. Under aerobiosis, deletion in rpoS resulted in a 1.5-fold increase in arcA transcription (not shown). Under microaerobic conditions, deletion of rpoS had no effect on arcA transcription during exponential growth. However, after transition into stationary phase, only a minor decrease in arcA transcription was observed in the ΔrpoS strain and arcA transcription remained at a high level (Fig. 5).

DISCUSSION

From the equation describing the most preferred and efficient Salmonella metabolism, C₆H₁₂O₆ + O₂ → 6CO₂ + 6H₂O, it is clear that the presence of an electron acceptor is equally important as the presence of organic...
carbon. Although *Salmonella* can also obtain energy from fermentation, this is much less efficient than respiration. Therefore if either glucose or oxygen is in short supply, *Salmonella* will restrict its metabolism and enter the stationary phase. Many studies have focused on carbon starvation (O'Neal *et al.*, 1994; Seymour *et al.*, 1996; Zgurskaya *et al.*, 1997) or oxygen metabolism (Iuchi & Weiner, 1996; Nystrom *et al.*, 1996) without attempts to link these two issues. Only Nystrom *et al.* (1996) noticed that proteins induced during glucose starvation and anaerobiosis are strikingly similar.

Using transcriptional fusions and bacterial cultures in aerobic and microaerobic conditions we have shown that *rpoS* transcription is upregulated by the presence of oxygen and modulated by the activity of the respiratory chain. Our results demonstrate that in the presence of oxygen, a few hours after maximal *rpoS* or *arcA* transcription, metabolism rapidly declines. Under aerobic conditions, we did not prove any significant interaction between NuoG, CydA, ArcA and RpoS.

Under microaerobiosis, we observed and characterized complex interactions between proteins essential for respiration or its control, and *rpoS* transcription. In the *nuoG* mutant, *rpoS* transcription was suppressed. NuoG is a subunit of NADH dehydrogenase I which catalyses the oxidation of NADH to NAD and the reduction of ubiquinone (Ub) to UbH$_2$. (Zambrano & Kolter, 1993). If NuoG activity is inactivated, either the NADH: NAD ratio is increased (due to the accumulation of NADH) or the UbH$_2$:Ub ratio is decreased. Both can be used as a signal for RpoS downregulation; however, as RpoS is a cytoplasmic protein it is more probable that changes of the NADH: NAD ratio occurring directly in the cytoplasm are used as a signal for *rpoS* regulation. Consistent with such a conclusion is the situation during aerobic carbon starvation, when the NADH: NAD ratio is likely to decrease (Iuchi *et al.*, 1994), resulting in increased *rpoS* transcription. Under conditions when oxygen becomes the limiting factor of metabolism, NADH levels increase, leading to the suppression of *rpoS* transcription. This is supported by our results showing that transcription of *rpoS* is reduced under microaerobiosis and also in the *nuoG* mutant, where the NADH level is likely to be increased. Unfortunately, we were unable to influence *rpoS* transcription by the direct addition of chemicals which may affect respiration. However, NADH can be quite unstable at 37 °C in LB broth cultures, and vanillylnonanamide and dicyclohexylcarbodiimide have been shown to be effective on NADH-ubiquinone reductase in membrane preparations (Yagi, 1987, 1990). The question remains to what extent these chemicals can be transported into *S. typhimurium* cells to be effective as respiratory inhibitors. Despite this we suggest that besides previously described signals for RpoS regulation such as ppGpp and cAMP (Lange *et al.*, 1995; Loewen *et al.*, 1998), the NADH: NAD ratio may further contribute to regulation of RpoS expression, mainly at the level of transcription.

Less clear is the explanation for increased transcription of *rpoS* in the *cydA* and *ΔarcA* mutants. As *rpoS* is upregulated in these mutants, activities of CydA and ArcA under microaerobiosis in the wild-type cells lead to the downregulation of *rpoS*. This means that as long as respiration or any other enzymic reaction catalysed by CydA or controlled by ArcA is possible, *rpoS* transcription is decreased. Conversely, the fact that deletion in *rpoS* results in increased *arcA* transcription in the stationary phase, means that when *rpoS* is finally transcribed and expressed, it actively suppresses *arcA* transcription (Fig. 3). Presumably, this would decrease the metabolic rate with two main consequences – to preserve internal energy resources for later recovery out of stationary phase (Siegele & Kolter, 1992; Kolter *et al.*, 1993; Siegele *et al.*, 1996) and to reduce production of toxic oxygen radicals (Iuchi & Weiner, 1996; Nystrom *et al.*, 1996). Under microaerobiosis, the ArcA and RpoS regulons thus mutually interact in finding the most appropriate mode of bacterial metabolism.

Interestingly, microaerobic *rpoS* transcription levels in the *cydA* and *ΔarcA* mutants were similar to the *rpoS* transcription level in the wild-type strain under aerobiosis, i.e. deletion of either of these genes resulted in an aerobic level of *rpoS* transcription even in microaerobic conditions (see Figs 1 and 4). When CydA is inactivated, remaining oxygen is not scavenged and this can serve as a signal for *rpoS* increased transcription. Because CydA is inactivated, remaning oxygen is not scavenged and this can serve as a signal for *rpoS* increased transcription. Because *cydA* is suppressed in *arcA* mutants (Rameiser *et al.*, 1996; Cotter *et al.*, 1997), the same explanation may also hold for the *arcA* mutant. However, it has been shown that CydA is important in the generation of an unknown signal for the ArcB sensor kinase of ArcA (Iuchi *et al.*, 1996b), thus playing a role in the control of ArcA expression. The same metabolite could suppress *rpoS* transcription. Such a hypothesis might be supported by recent findings that *rpoS* in *E. coli* responds to homoserine lactone (Huisman & Kolter, 1994), one of the possible metabolites involved in bacterial density-dependent signalling (Fuqua *et al.*, 1994). Furthermore, in *Pseudomonas* and *Erwinia* a link between carbon starvation control and quorum sensing has already been demonstrated (Cui *et al.*, 1995; Latifi *et al.*, 1996). A such a hypothesis is further strengthened by the fact that upregulation in both *rpoS* and *arcA* occurred in cultures with OD$_{600}$ values between 0·4 and 0·6, regardless of the fact that under microaerobic conditions this was nearly the point of entry into stationary phase while under full aerobiosis, growth continued until a nearly 10-fold higher culture density was reached. Possible active downregulation of metabolism by sensing of secondary metabolites in *Salmonella* has been already suggested (Barrow *et al.*, 1996; Zhang-Barber *et al.*, 1997) and recently, a metabolite produced by *Salmonella* which cross-reacts with the quorum-sensing system of *Vibrio harveyi* was detected (Surette & Bassler, 1998; Surette *et al.*, 1999). The peak in metabolite production described by Surette & Bassler (1999) was positioned just prior to entry to stationary phase, similar to our observation of maximal transcription of *rpoS* and *arcA*. However, it remains to be determined whether any *Salmonella*
specific secondary metabolite signalling occurs during transition into stationary phase.

ACKNOWLEDGEMENTS

This work was supported by a grant of the Grant Agency of the Czech Republic (524/98/1089) and an EU FAIR grant (PL98-4006).

REFERENCES


Received 2 August 2000; revised 13 November 2000; accepted 17 November 2000.