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

Veterinary Research
Institute, 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, Δ *arcA* and Δ *rpoS* 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-

Abbreviation: RLU, relative luminescence units (luminescence per OD₆₀₀ unit).

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.*, 1998).

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 $\Delta rpoS$ and $\Delta arcA$, and a wild-type *S. typhimurium* strain. This allowed us to demonstrate that control of carbon starvation and control of aerobiosis/anaerobiosis are two complementary sites of bacterial response to stress conditions.

METHODS

Bacterial strains and growth conditions. *S. typhimurium* F98 NaI^R (resistant to nalidixic acid) and its isogenic mutants *nuoG::Km* and *cydA::TnpH* have been described previously (Zhang-Barber *et al.*, 1997). Construction of $\Delta rpoS$ and $\Delta arcA$ 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 5×10^3 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-nonanamide (50 µM), and dicyclohexylcarbodiimide (50 µM) on *rpoS* transcription was investigated. All these chemicals were from Sigma.

Construction of transcriptional *luxAB* fusions and lumino-metry. 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 electrotransformation of *E. coli* 17.1λpir (*E. coli* Pulser, 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 NaI^R. As *S. typhimurium* does not support the replication of R6K ori pir⁻ plasmids (i.e. pNQ705L), the Cm^R (chloramphenicol-resistant)/NaI^R 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 luminometry, 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 *Xba*I and *Xho*I (primers contained *Xba*I or *Xho*I sequences

Table 1. Primers

cydAF/cydAR PCR yielded positive amplification in the wild-type strain but not in the *cydA* mutant. Tn insertion in *cydA* mutant was confirmed by cydAF/IS50 PCR, which was positive in the mutant only. nuoGF/nuoGR PCR resulted in a 581 bp product in the wild-type strain and a 1791 bp product in the mutant strain (581 bp of the *nuoG* sequence plus 1210 bp of the kanamycin gene cassette). Primers rpo1–rpo4 and arc1–arc4 were used for construction of a deletion in *rpoS* and *arcA*, respectively. Primers rpo1 and rpo4 contained *Xba*I or *Xho*I 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; *Xba*I and *Sac*I overhangs (underlined) enabled cloning into pNQ705L. The luxAR primer is derived from the sequence of the *luxA* gene. rpo1/luxAR (arc1/luxAR) PCR was used for the verification of insertion of the transcriptional fusion into the final recipient strain.

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

respectively; see also Table 1), purified again with the QIAquick Gel Extraction kit and ligated into *Xba*I/*Xho*I-linearized pDM4. After ligation (T4 DNA ligase, US Biochemicals) the recombinant plasmid was electrottransformed into *E. coli* 17.1λ*pir* and Cm^R colonies were tested by PCR for the presence of the deleted *rpoS* gene sequence. Recombinant pDM4Δ*rpoS* was conjugated into *S. typhimurium* F98 Nal^R. As *S. typhimurium* does not encode the *pir* protein, the only Cm^R/Nal^R 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 Tn*phoA* 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 Δ*rpoS* and Δ*arcA* 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 inac-

tivation 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

Fig. 1. Transcription of *rpoS-luxAB* (■) and *arcA-luxAB* (▲) in the wild-type *S. typhimurium* F98 NaI^R strain grown under full aerobiosis. +, OD₆₀₀.

amino acid exchange, Met-53 to Ile-53, as well as a deletion from Asp-54 to Lys-103, inclusive. The genotype of the mutant was confirmed phenotypically by sensitivity to toluidine blue (Iuchi *et al.*, 1990a).

The *nuoG*, *cydA* and *arcA* mutants grew more slowly than the wild-type strain, in both aerobic and microaerobic conditions; the defect was more obvious in the microaerobic cultures (not shown). The $\Delta rpoS$ mutant behaved differently from the wild-type strain and the other mutants. In the exponential phase it grew faster than the wild-type, probably due to the reduced competition of RpoD and RpoS for a limited amount of core RNA polymerase (Farewell *et al.*, 1998). Later, the $\Delta rpoS$ mutant was unable to reach the same culture density as the wild-type strain (not shown), a finding compatible with previous observations of an important function of RpoS in stationary phase (Fang *et al.*, 1992; O'Neal *et al.*, 1994; Loewen *et al.*, 1998). During growth of the culture, after the initial decrease in pH of the broth from 7.6 to pH 6.6 (microaerobic) or 7.3 (aerobic), an increase in pH was observed; the final pH reached was 7.0 under microaerobiosis and 8.8 under aerobiosis. The redox potential of initial LB broths was around +100 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

Fig. 2. Transcription of *rpoS-luxAB* (■) and *arcA-luxAB* (▲) in the wild-type *S. typhimurium* F98 NaI^R strain grown under microaerobiosis. +, OD₆₀₀.

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 $\Delta arcA$ 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 $\Delta arcA$ mutants and the wild-type strain

Fig. 3. Influence of mutations in the respiratory chain on *rpoS* transcription in *S. typhimurium* grown under full aerobiosis. ■, Wild-type F98 Nal^R strain; ●, *arcA* mutant; □, *nuoG* mutant; △, *cydA* mutant. The shift in the peak of transcription in the *nuoG*, *cydA* and *arcA* mutants was caused by their slower growth.

Fig. 4. Influence of mutations in the respiratory chain on *rpoS* transcription in *S. typhimurium* grown under microaerobiosis. ■, Wild-type F98 Nal^R strain; ●, *arcA* mutant; □, *nuoG* mutant; △, *cydA* mutant. The shift in the peak of transcription in time in the *cydA* and *arcA* mutants was caused by their slower growth. The *nuoG* mutant reached stationary phase on day 2; the *cydA* and *arcA* mutants entered stationary phase when the cultures were 3 d old. Note the change in scale after 24 h on the x axis.

was virtually identical (Fig. 3). The shift of the peak of maximal *rpoS* transcription in the mutants was caused only by their slower growth. In contrast, in microaerobically grown cultures, *rpoS* was suppressed in the *nuoG* mutant whereas in the *cydA* and *arcA* mutants *rpoS* was upregulated (Fig. 4). The maximal *rpoS* transcription rate observed in the *cydA* and Δ *arcA* mutants was more than twofold higher than in the wild-type strain. Transcription of *rpoS* in the *cydA* mutant could be observed even in 21-d-old cultures, when *rpoS* transcription in both the wild-type strain and the *nuoG* mutant was nearly zero (not shown). Maximal levels of

Fig. 5. Influence of the *rpoS* deletion on *arcA* transcription in *S. typhimurium* grown under microaerobiosis. ▲, Wild-type F98 Nal^R strain; ○, Δ *rpoS*.

rpoS transcription, reached microaerobically, in *cydA* and Δ *arcA* mutants corresponded to the level of transcription observed in the wild-type strain under aerobic conditions (see Figs 1 and 4).

We also tested direct addition of chemicals which could influence the NADH/NAD ratio in the bacterial cells. Dinitrophenol is an uncoupler of oxidative phosphorylation, vanillynonanamide is an analogue of capsaicin, and together with dicyclohexylcarbodiimide these chemicals have been shown to specifically inhibit membrane preparations of NADH-ubiquinone reductase (Yagi, 1987, 1990). None of these chemicals, nor direct addition of NADH into nutrient broth, influenced *rpoS* transcription when used at 50 μ M concentration (1 mM for NADH); all of them (except for NADH) started to affect growth when present at 100 μ M or higher (not shown).

Interaction of RpoS and ArcA

Increased *rpoS* transcription in the Δ *arcA* 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 Δ *rpoS* strain and *arcA* transcription remained at a high level (Fig. 5).

DISCUSSION

From the equation describing the most preferred and efficient *Salmonella* metabolism, $C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O$, 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 UbH₂ (Zambrano & Kolter, 1993). If NuoG activity is inactivated, either the NADH:NAD ratio is increased (due to the accumulation of NADH) or the UbH₂: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 Δ *arcA* 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. 5). 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 Δ *arcA* 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 suppressed in *arcA* mutants (Ramseier *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.*, 1990b), 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). Such a hypothesis is further strengthened by the fact that upregulation in both *rpoS* and *arcA* occurred in cultures with OD₆₀₀ 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.

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