Evidence that \textit{rscC}, a Gene in the \textit{rpoE} Cluster, Has a Role in Thiamine Synthesis in \textit{Salmonella typhimurium}

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In \textit{Salmonella typhimurium}, the genetic loci and biochemical reactions necessary for the conversion of aminoimidazole ribotide (AIR) to the 4-amino-5-hydroxymethyl-2-methyl pyrimidine (HMP) moiety of thiamine remain unknown. Preliminary genetic analysis indicates that there may be more than one pathway responsible for the synthesis of HMP from AIR and that the function of these pathways depends on the availability of AIR, synthesized by the purine pathway or by the purF\textsuperscript{-}independent alternative pyrimidine biosynthetic (APB) pathway (L. Petersen and D. Downs, J. Bacteriol. 178:5676–5682, 1996). An insertion in \textit{rscB}, the third gene in the \textit{rpoE} \textit{rscABC} gene cluster at 57 min, prevented HMP synthesis in a \textit{purF} mutant. Complementation analysis demonstrated that the HMP requirement of the \textit{purF} \textit{rscB} strain was due to polarity of the insertion in \textit{rscC} on the downstream \textit{rsc} gene. The role of \textit{RseC} in thiamine synthesis was independent of \textit{rpoE}.

An efficient metabolism demands the coordination of many biochemical pathways and processes. Biochemical pathways, particularly those sharing metabolites, must interact at many levels to achieve such coordination. We use the low-flux thiamine biosynthetic pathway in \textit{Salmonella typhimurium} as a model system for addressing interactions between pathways. Since thiamine is essential for growth, the difference between growth and no growth can reflect subtle changes in flux through this pathway, thus providing a level of sensitivity that would not be possible with biosynthetic pathways requiring high carbon flux. In efforts to further characterize the coordination of purine and thiamine biosynthetic pathways, we have identified several genetic loci involved in thiamine synthesis (8, 16). Through this analysis, we have demonstrated an involvement of at least four metabolic pathways, in addition to de novo purine synthesis, in the formation of thiamine and demonstrated examples of metabolic cross talk and metabolite sharing in this system (8, 17).

Thiamine consists of an 4-amino-5-hydroxymethyl-2-methyl pyrimidine (HMP) moiety and a 4-methyl-5(\textit{β}-hydroxethyl) thiazole (THZ) moiety which are independently synthesized and phosphorylated prior to the formation of thiamine pyrophosphate (TPP), the coenzymic form of thiamine (21). The focus of our work is to understand the pathways involved in synthesis of the HMP moiety of thiamine. HMP can be generated via de novo purine biosynthesis, with aminoimidazole ribotide (AIR) as the last shared intermediate (14, 21), or via the alternative pyrimidine biosynthetic (APB) pathway (7, 17). Our current view of the metabolic inputs to HMP synthesis are depicted in Fig. 1. It is important to emphasize that while for simplicity we view these inputs as pathways, they may utilize metabolites generated by side reactions of other enzymes of known function.

Genetic data has led us to propose that the conversion of AIR to HMP can occur by more than one pathway and that the relative activities of these pathways depend on the availability of AIR (16). By this model, mutants blocked in the low-flux pathway would be prototrophic in a Pur\textsuperscript{+} background due to flux through the de novo purine biosynthetic pathway. Such mutations would be expected to cause a thiamine requirement in a purF\textsuperscript{-} mutant background, due to reduced AIR formation, and further, a block in \textit{purF} would be expected to restore thiamine synthesis in these strains. Mutations in two genetic loci that meet these criteria have been identified: \textit{apbC} and \textit{apbE} (2, 16).

We report here that \textit{rscC}, the fourth gene in the \textit{rpoE} cluster (6, 13), falls in the class of loci required for \textit{purF}-independent synthesis of the pyrimidine ring of thiamine and suggest that this requirement is in the conversion of AIR to HMP. Data presented here demonstrate that the role of \textit{RseC} in thiamine synthesis is independent of \textit{rpoE}.

\textbf{Initial observations.} In the course of other work we identified an insertion mutation (MudJ) that prevented thiamine synthesis in a \textit{purF} mutant (17). Linkage analysis determined that this MudJ insertion was not linked to known \textit{thi} loci at 10, 46, or 90 min on the \textit{S. typhimurium} chromosome. By using Mud-P22 technology (3) and dideoxy sequencing, the DNA sequences on each side of the MudJ insertion were determined. Analysis of the resulting sequence with a BLASTX computer search (1, 10) determined that the MudJ insertion was in an open reading frame (ORF) homologous to \textit{RseB} in \textit{Escherichia coli}. Sequence data supporting the designation of this insertion as a lesion in \textit{rscB} (\textit{rscB1}::MudJ) are shown in Fig. 2. An insertion in \textit{rscB} reduces thiamine synthesis in a \textit{purF} mutant background. We confirmed that the \textit{rscB1}::MudJ insertion affected thiamine synthesis by several criteria. Unlike the parental strain DM1936 (\textit{purF2085}), strain DM857 (\textit{purF2085 rscB1}::MudJ) was unable to grow in the absence of thiamine in minimal glucose medium supplemented with adenine (0.5 mM). As shown in Fig. 3, a wild-type growth rate (i.e., 0.5 to 0.6 h\textsuperscript{-1}) was restored by the addition of 100 nM exogenous thiamine. The \textit{rscB1}::MudJ caused no discernible
phenotype in a wild-type background, thus placing this mutant in a growing class of conditional thiamine auxotrophs.

Further characterization of the thiamine requirement of strain DM857 (purF2085 rseB1::MudJ) showed that HMP was sufficient to satisfy the thiamine requirement of this strain. Recent work has shown that the conversion of AIR to HMP is likely to occur by more than one pathway and that the relative activities of these pathways depend on the availability of AIR (16). Consistent with this model, an insertion mutation in the purE gene restored thiamine synthesis in a subclass of mutants defective in thiamine synthesis. In the case of apbC, this effect was shown to be mediated through an accumulation of AIR (16). When a purE mutation was introduced into strain DM857 (purF2085 rseB1::MudJ), the resulting strain, DM2174 (purF2085 rseB1::MudJ purE884::MudJ), regained the ability to grow in the absence of thiamine (data not shown). This result was consistent with the rseB1::MudJ mutation causing a defect in the conversion of AIR to HMP.

To eliminate the possibility that rseB1::MudJ caused an increased cellular requirement for thiamine as opposed to the inability to synthesize the vitamin, TPP pools were measured in relevant mutant backgrounds. Total TPP pools were determined by a thiachrome derivatization assay (20) in strains DM1936 (purF2085) and DM857 (purF2085 rseB1::MudJ), grown to mid-log phase in minimal gluconate medium supplemented with adenine (0.5 mM) and limiting thiamine (10 nM). Levels of TPP from a representative experiment were 36 pmol/mg (dry weight) for DM1936 (purF2085) and 22 pmol/mg (dry weight) for DM857 (purF2085 rseB1::MudJ). Strain DM857 consistently had TPP levels that were 40 to 50% of those of the isogenic strain DM1936, supporting the conclusion that rseB1::MudJ affected synthesis and not the cellular requirement for thiamine. Consistent with its growth phenotype, strain DM2174 (purF2085 rseB1::MudJ purE884::MudJ) contained a TPP pool of 40 pmol/mg (dry weight) in the same experiment. Taken together, these results were consistent with the rseB1::MudJ insertion affecting the conversion of AIR to HMP, either directly or indirectly.

The phenotype of an rseB1::MudJ insertion is due to polarity on rseC. RseB is encoded by the third gene in the rpoE cluster of E. coli (6, 13). In at least three other organisms, Pseudomonas aeruginosa (19), Azotobacter vinelandii (12), and Photobacterium sp. strain SS9 (5), a gene cluster with a similar arrangement has been identified. In each case, the first gene of the cluster encodes an alternative sigma factor belonging to the extracytoplasmic function subfamily (11). Because of the possibility that this gene cluster formed an operon, complementation analyses were performed to determine if polarity on rseC was responsible for the thiamine-related phenotypes caused by the rseB1::MudJ insertion (Fig. 2). Two expression vectors, one containing rseB and one containing rseC, were independently introduced into DM857 (purF2085 rseB1::MudJ), and the resulting strains were scored for thiamine-independent growth on gluconate adenine medium. The strain containing pLC203 (rseB) remained unable to grow in the absence of thiamine. In contrast, the strain with pAP72 (rseC) grew in the absence of thiamine, and when tested on solid medium, this growth was indistinguishable from that of the control strain DM1936 (purF2085). From these results we concluded that the rseB1::MudJ insertion disrupted thiamine synthesis by preventing
transcription of rseC, the fourth gene in the rpoE gene cluster that encodes an inner membrane protein (6, 13).

This result was reminiscent of a report on Photobacterium sp. strain SS9 that described a barotolerance phenotype associated with an insertion in ORF3 (RseB homolog) in the rpoE cluster that was complemented by ORF4 (RseC homolog) alone (5). To reflect our finding, the relevant genotype of mutants carrying the rseB1::MudJ insertion is referred to here as rseC.

The requirement for RseC in thiamine synthesis is independent of rpoE. Because of the conserved gene organization of the rpoE gene cluster in several organisms, it has been suggested that the downstream ORFs (including the RseC homologs) are involved in regulation of the respective sigma factors (12). Recently, ErE was determined to be regulated by RseAB in E. coli (6, 13) and MucAB in P. aeruginosa (19, 22), but regulation of ErE activity by RseC has remained unclear (6, 13). We considered that the role of RseC in thiamine synthesis could be dependent or independent of ErE activity. In the first scenario, subnormal levels of ErE could result in a thiamine requirement if one or more of the genes needed for HMP synthesis required this sigma factor for transcription. Alternatively, RseC could contribute to thiamine synthesis independently of its possible role in regulating ErE activity.

The simple scenario that ErE was required for gene expression predicted that a null mutation in rpoE would result in a thiamine requirement in a purF mutant. To test this, we transduced an E. coli rpoE::Ω/Cm' insertion into an S. typhimurium purF strain via an intermediate mutS mutant as has been described (15). Isogenic strains DM2641 (purF2085 rpoE::Ω/Cm') and DM2642 (purF2085 rpoE::Ω/Cm' rseB1::MudJ) were constructed, and their thiamine-independent growth was compared to that of parent strain DM1936 (purF2085).

FIG. 2. Position of the rseB1::MudJ insertion in the rpoE gene cluster. The physical organization of the rpoE gene cluster in S. typhimurium and E. coli at 57 min is represented. The sequences flanking the MudJ insertion were compared with the deduced amino acid sequence of RseB from E. coli. Identical amino acids are shown in bold, and position numbers reflect the numbering of amino acid sequence of RseB in E. coli. The complete ORFs contained in various plasmids used for complementation analysis are shown.

FIG. 3. The rseB1::MudJ insertion causes an APB phenotype. Growth cultures were performed as described previously (17). DM1639 (purF2085) (A) and DM857 (purF2085 rseB1::MudJ) (B) were grown at 37°C. Open symbols, minimal gluconate medium supplemented with 0.5 mM adenine; closed symbols, minimal gluconate medium supplemented with 0.5 mM adenine and 100 nM thiamine.

These results supported an rpoE-independent role for RseC in thiamine synthesis.

In order to further test the hypothesis that the role of RseC in thiamine synthesis was independent of ErE, the rpoE operon was deleted by the method of Bochner et al. (4). Strains with deletions removing material between the nadB gene and the zff-8075::Tn10d(Tc) element (located downstream of the rpoE operon) were isolated. Strains containing such deletions required nicotinic acid for growth (NadB+) and had a noticeably reduced growth rate at 30°C (rpoE). The absence of RpoE was confirmed by the inability of these deletion mutants to direct transcription from an ErE-dependent rpoH P3-lacZ fusion (81 versus 10 U for the wild type and the ΔrpoE strain, respectively). Restoration of wild-type growth in these deletion...
strains was obtained when the entire rpoE operon was provided in trans (pAP25) and nicotinic acid was added to the medium. Significantly, this result indicated that any deleted genetic material upstream of nadB or downstream of the rpoE operon was not responsible for the observed phenotypes.

A representative nadB-rseC deletion (DE3645), in a purF background, was chosen for analysis, and results are presented in Table 1. As expected, the deletion strain DM2464 (purF2085 DE3645 ΔnadB-rseC) was unable to grow unless thiamine was added to the medium (Table 1). Introduction of a plasmid carrying the entire rpoE operon (pAP25) or rseC alone (pAP72) restored growth (i.e., strains were able to synthesize thiamine) (Table 1). In contrast, introduction of a plasmid carrying only rseB (pLC203) had no effect on the growth of DM2464 (purF2085 DE3645 ΔnadB-rseC) (data not shown).

The ability of the plasmid carrying only rseC to restore thiamine synthesis in a strain with the entire rpoE operon deleted clearly demonstrated that the role of rseC in thiamine synthesis was not mediated through ErE.

Two additional points arose from the results in Table 1. First, strains defective in rpoE (i.e., DM2464 [purF2085 DE3645 ΔnadB-rseC]) and DM2570 [purF2085 DE3645 ΔnadB-rseC/pAP72] showed a significant lag (>4 h) prior to beginning logarithmic growth. A similar lag was detected with strains carrying the rpoE::Ω/Cm cassette described above. The eventual growth of these strains did not appear to be due to the accumulation of an additional mutation(s), since a similar lag phase was observed after these cultures were reinoculated into fresh medium. Second, the growth phenotype of the rpoE deletion strain DM2464 was significantly more severe than that of the rseB1::MudJ strain [DM857 (purF2085 rseB1::MudJ)], as indicated by growth rates of 0.1 and 0.25, respectively, in the absence of thiamine. This difference could reflect the residual transcription of rseC in DM857, or it may be due to the additive effect of an rpoE mutation disrupting cellular metabolism combined with the loss of rseC.

Homologs of RseC have been identified in other organisms and are generally encoded by genes found in clusters analogous to the E. coli rpoE cluster. In Photobacterium sp. strain SS9, a homolog of RseC is required for psychrotolerance (5). More recently, MucC from P. aeruginosa and MucC from S. typhimurium have been identified as RseC homologs and may be involved in alginate formation (12, 19). In each of these cases it remains unclear whether these homologs act as regulators of the associated sigma factor. However, at least three homologs of RseC that are not located near a sigma factor have been described: rnfF, which is required for nitrogen fixation in Rhodobacter capsulatus (18), and YS50 and YS89 from Haemophilus influenzae, which do not have known functions (9). The existence of RseC homologs that are not linked to rpoE in other organisms is consistent with our identification of an rpoE-independent role for RseC in metabolism.

The role of RseC in thiamine synthesis remains unknown. The direct participation of RseC in a catalytic reaction for thiamine synthesis seems unlikely considering its identification as a transmembrane, inner membrane protein (6, 13). Instead, a more attractive model for the involvement of RseC in thiamine synthesis involves the interaction of other proteins with RseC. Perhaps the interaction between RseC and one or more enzymes is required for significant synthesis of the pyrimidine moiety of thiamine. The amino acid similarity between RnfF (R. capsulatus) and RseC supports this model, as does the recent identification of a lipoprotein from S. typhimurium that also has considerable homology with RnfF and is required for optimal thiamine synthesis (2). RnfF has been postulated to promote a membrane-associated complex responsible for transfer of reducing power to nitrogenase during nitrogen fixation (18). Further, the role of RseC as a protein complex anchor is consistent with its putative role as a regulator of ~r^E levels through interactions with RseA and RseB.

This work describes a clear nutritional phenotype for rseC mutants of S. typhimurium. These experiments represent the first demonstration that eliminating RseC disrupts physiology in a way that is independent of its proposed role in regulating ErE activity. Taken together, these results are consistent with at least two roles for RseC in the cell: (i) RseC may act as a regulator of ErE, perhaps through RseAB, and (ii) RseC is also involved in synthesis of the pyrimidine ring of thiamine (HMP). Further definition of the role that RseC and other membrane proteins play in thiamine synthesis has the potential to increase our understanding of how the cell can couple physiological processes and global regulatory systems.

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REFERENCES


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Table 1. RpoE is not required for thiamine synthesis

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<th>Strain</th>
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a Strain genotypes and plasmids are as follows: DM1936, purF2085; DM857, purF2085 rseB1::MudJ; DM2464, purF2085 DE3645ΔnadB-rseC; pAP25, Kan^R rpoE rseABC in pBR322; pAP72, Ap^R rseC in pTc29A.

b Strains were grown in minimal gluconate media with the indicated supplements in a 37°C water bath with shaking (250 rpm). Ade, adenine; Nic, nicotinic acid; Thi, thiamine.

c Specific growth rate (μ) was determined by the equation ln(X/X0)/T, where X is A_650, X_0 is A_650 at time zero, and T equals time (in hours).

d A_650 at time zero was between 0.02 and 0.05. A_650 was determined after 20 h of incubation.

Strains without a functional RpoE had a significant lag phase (>4 h) prior to logarithmic growth.
6508 NOTES J. BACTERIOL.


