Ectoine Biosynthesis in *Mycobacterium smegmatis*

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Mycobacterium smegmatis is a commonly used mycobacterial model system. Here, we show that *M. smegmatis* protects itself against elevated salinity by synthesizing ectoine and hydroxyectoine and characterize the phenotype of a nonproducing mutant. This is the first analysis of *M. smegmatis* halotolerance and of the molecular mechanism that supports it.

*M*ycobacterium smegmatis, an actinobacterial species, is commonly found in soil, marine, and freshwater environments ([20]). Due to its nonpathogenic nature and relatively high growth rate, *M. smegmatis* is used as a model mycobacterial system, especially for the study of pathogenic mycobacteria, including *Mycobacterium tuberculosis*. The natural habitat of *M. smegmatis*, however, is very different from that of obligatory pathogens; to thrive in nature, *M. smegmatis* must be capable of adapting to fluctuations in various environmental conditions. This report focuses on *M. smegmatis* adaptation to high salinity.

Two different strategies for survival in salty environments are known. Confined mainly to extreme halophiles, one strategy is the intracellular accumulation of inorganic salts to counterbalance the high extracellular salt concentrations ([19], [25]). A second strategy, typical in moderate halophiles and halotolerants, is the synthesis of low-molecular-weight, organic osmolytes that counterbalance the extracellular osmotic pressure but do not increase the ionic strength of the cytoplasm ([19], [26]). These molecules, termed compatible solutes, are usually zwitterionic or uncharged and are highly soluble in water ([26]). Commonly found compatible solutes in bacteria are ectoine and its hydroxylated derivative, hydroxyectoine ([7], [10], [11], [21], [24]). The ectoine biosynthetic enzymes are encoded by three genes: *ectA*, *ectB*, and *ectC* ([22]) ([Fig. 1]). An additional enzyme, encoded by *ectD*, is often found in many ectoine-producing species and is responsible for the conversion of ectoine to hydroxyectoine ([11]). These genes are highly conserved in diverse bacterial species residing in environments of high or changing salinity, and it was shown in several cases that ectoine and hydroxyectoine biosyntheses were strongly induced under conditions of high salt concentrations ([2], [3], [4], [6], [8], [15], [16]) and that ectoine-deficient mutants were salt sensitive ([5], [9], [23], [29]). There is also evidence for induction of the *ect* genes at elevated temperatures ([2], [8]) and for the ability of ectoine and, to a greater extent, of hydroxyectoine to protect proteins against thermal denaturation ([1], [12], [13], [14], [17]). In addition, ectoine can effectively protect the skin of humans against dehydration and against the carcinogenic effect of long-term exposure to sunlight. Ectoine is, therefore, commonly found in sunscreens and body lotions, which results in an immense industrial importance of this chemical ([21]).

The annotated genome sequence of *M. smegmatis* strain MC^{2}155 indicates the presence of a putative hydroxyectoine biosynthesis gene cluster ([18], [27], [28]). To examine whether this cluster is transcribed, a quantitative reverse transcription-PCR (qRT-PCR) analysis was carried out. NaCl was added to exponentially growing *M. smegmatis* cultures (30°C; 7H9 media containing 0.4% glycerol and 0.05% Tween 80), and samples were collected over the course of 2 h. For each sample, total RNA was purified, and qRT-PCR was carried out using specific *ectB* primers (see Table S1 in the supplemental material). Control cultures were kept at 30°C in the original growth medium without NaCl addition. An approximately 40- to 60-fold increase in the *ectB* mRNA level was detected in cultures containing 0.5 and 1 M NaCl, suggesting a strong induction in response to an increase in salinity ([Fig. 2A]). In contrast, no induction was observed in control cultures and in cultures that were grown at 42°C in the absence of added salt. Therefore, high salinity, but not high temperatures, leads to induction of the *M. smegmatis ectB* gene. Induction in response to an increase in salinity could also be observed using green fluorescent protein (GFP) as a reporter protein ([Fig. 2B]). To test whether the *ect* gene cluster comprises an operon, cDNA was purified and analyzed by PCR using primers specific for the DNA junctions between *ectA* and *-B*, *ectB* and *-C*, and *ectC* and *-D*. Specific bands were clearly detected in samples taken from cultures incubated with NaCl, indicating that the *ect* gene cluster does indeed form an operon ([Fig. 2C]).

To test whether ectoine and hydroxyectoine are actually synthesized following an increase in salt concentration, *M. smegmatis* cultures were grown with and without salt at either 30 or 42°C, and samples were collected over the course of 4 h. From each sample, small molecules were extracted as described previously ([2]), and the amounts of ectoine and hydroxyectoine were analyzed by liquid chromatography-mass spectrometry (LCMS) using a Clarity Oligo-WAX column (100 by 4.6 mm, 300 Å). The results show that after the addition of salt, but not in its absence, hydroxyectoine gradually accumulated ([Fig. 2D]). Ectoine also accumulated in salt-containing cultures but to a level ∼100-fold lower than that of hydroxyectoine ([Fig. 2D], inset). Incubation at 42°C in the absence of salt did not lead to hydroxyectoine synthesis, further indicating that *M. smegmatis* produces ectoine in response to an increase in salinity but not in response to a temperature upshift.

To phenotypically analyze an ectoine-nonproducing mutant, the *ect* operon was deleted from the *M. smegmatis* chromosome using a recently developed gene knockout system. This system is
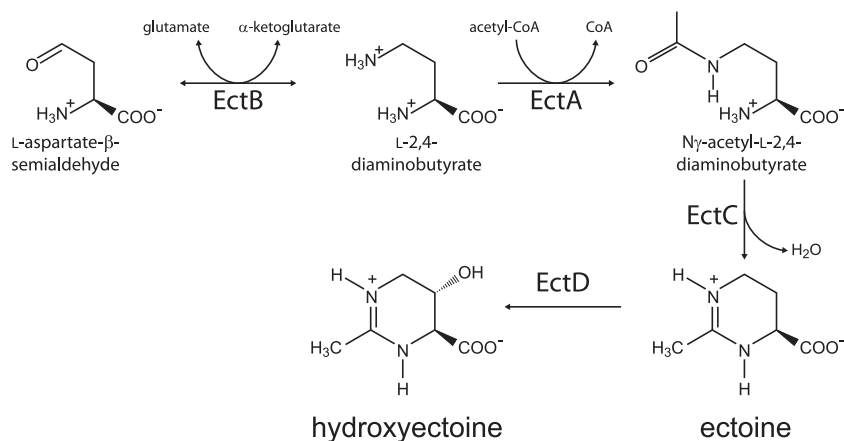
FIG 1 The biosynthetic pathway of ectoine and hydroxyectoine.

based on the use of plasmids pML2424 and pML2714 for the construction and selection of desired deletion mutants (see Fig. S1 in the supplemental material). Plasmid pML2424 allows directed insertion of a *gfp-hyg* cassette instead of a gene of interest in the *M.* *smegmatis* chromosome. Plasmid pML2714 enables excision of the *gfp-hyg* cassette from the chromosome via site-specific recombination (see Fig. S2 in the supplemental material). To delete the *ect* operon from the *M. smegmatis* chromosome, flanking regions
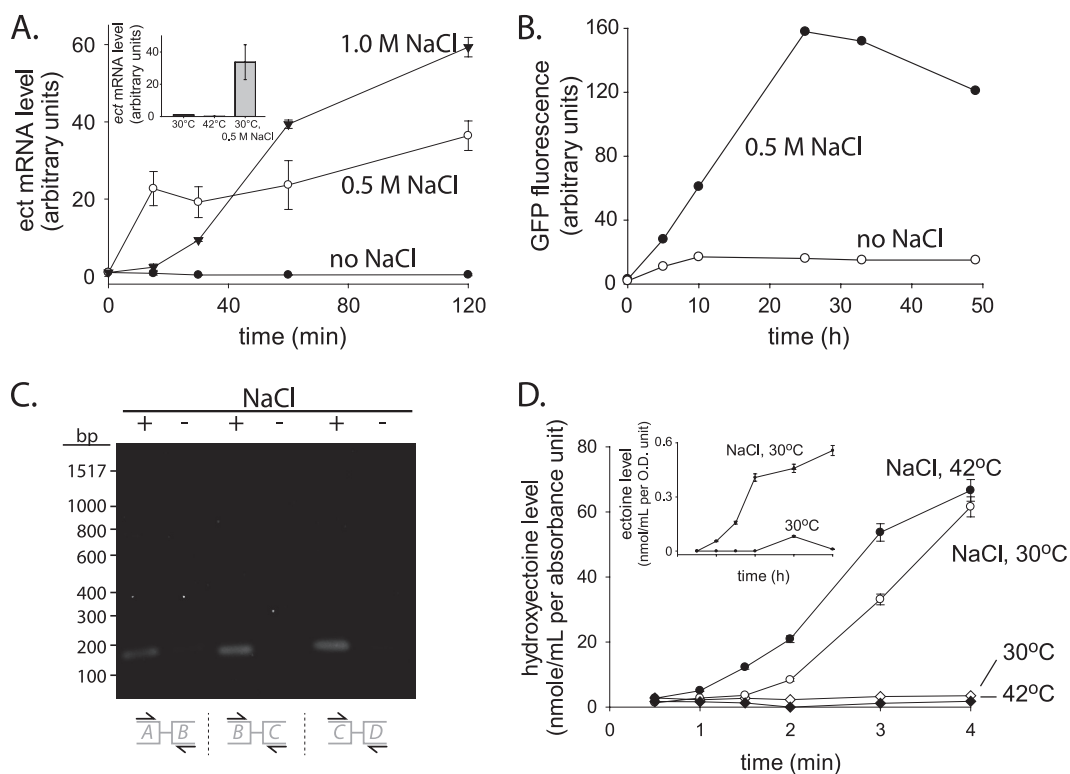

FIG 2 The *ect* biosynthetic pathway is induced at high salt concentrations. (A) Quantitative RT-PCR (qRT-PCR) analysis of the *ectB* gene was carried out (primers 1 and 2; see Table S1 in the supplemental material) for *M. smegmatis* cultures (optical density at 600 nm [OD_{600}] = 0.6) that were grown for 2 h at 30°C with 0, 0.5, and 1 M NaCl or without salt addition. *M. smegmatis sigA* was used as an internal reference gene (primers 3 and 4; see Table S1 in the supplemental material). A similar analysis was carried out for cultures that were incubated at 42°C without salt (inset). (B) *M. smegmatis* cultures (OD_{600} = 0.5) that express GFP under the transcriptional control of a putative *ectA* promoter (237 bp upstream of the *ectA* translation start site) were grown at 30°C with or without NaCl (0.5 M), and samples were collected at the indicated time points. Following cell lysis, GFP fluorescence was measured in lysates of equal protein concentrations. (C) Exponentially growing *M. smegmatis* cultures (30°C, OD_{600} = 0.5) were incubated for 2 h with or without NaCl (0.5 M). cDNA was purified and used as a template for PCR amplification reactions with primer pairs 5 and 6, 7 and 8, and 9 and 10 (see Table S1 in the supplemental material). (D) Hydroxyectoine levels as measured by LCMS analysis of samples collected at the indicated time points from cultures that were grown in 7H9 media with and without NaCl (0.5 M) at 30 or 42°C. Ectoine levels are plotted in the inset. The solute levels were determined using a standard curve of commercially available ectoine and hydroxyectoine (Sigma).
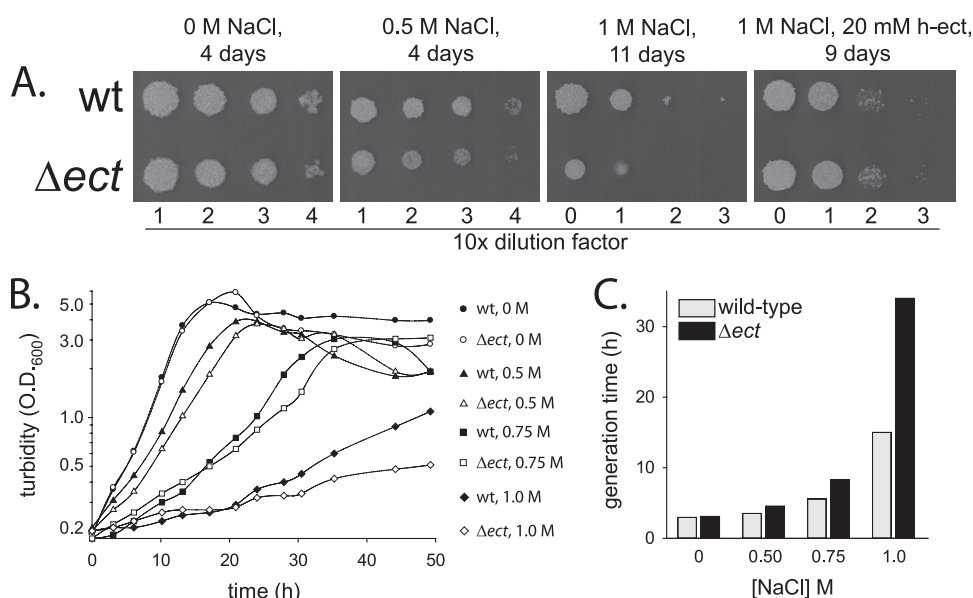
FIG 3 Osmosensitivity of a Δ*ect* mutant. (A) Spot tests of the wild-type (wt) strain and of the Δ*ect* mutant on 7H10 plates containing 0.4% glycerol and sodium chloride as indicated. In the right-hand panel, a plate containing both NaCl (1 M) and hydroxyectoine (h-ect) (20 mM) was used. (B) Growth curves of the wild-type strain (closed symbols) and of the Δ*ect* strain (open symbols) grown at 37°C in 7H9 liquid media with the addition of 0.5 (triangles), 0.75 (squares), and 1.0 M (diamonds) NaCl. No NaCl was added to control cultures (circles). (C) Generation times of the wild-type strain and the Δ*ect* mutant strain as calculated from the growth curves presented in panel B.

of the *M. smegmatis ect* operon were cloned before and after the *gfp-hyg* cluster (see Fig. S1 and S2A in the supplemental material), and isolation of the desired mutants was confirmed by PCR analysis and Southern blotting (see Fig. S2B and C in the supplemental material).

Halotolerance of a Δ*ect* mutant was examined initially by spot tests on plates containing 0, 0.5, or 1 M sodium chloride (Fig. 3A). No growth differences between the mutant and the wild type were observed in the absence of added salt. At 0.5 M salt, however, the mutant grew somewhat slower than the wild type, and at 1 M, it grew considerably slower. Addition of hydroxyectoine (20 mM) to the plates completely cured the growth defects of the mutant, indicating that despite its inability to synthesize ectoine, the mutant can accumulate the solute. The Δ*ect* growth phenotype could also be complemented by a plasmid expressing the *ect* operon (see Fig. S2 in the supplemental material). To better assess the phenotype of the Δ*ect* strain, its growth in liquid media containing 0, 0.5, 0.75, or 1 M NaCl was compared to that of the wild type (Fig. 3B). The Δ*ect* strain and the wild-type strain grew equally well in the absence of added salt, with generation times of 3.0 and 3.1 h, respectively (Fig. 3C). In comparison to the growth seen with the wild type, however, the growth of the mutant strain was suppressed to a greater extent in a salt-concentration-dependent manner (Fig. 3C). For example, at 0.5 M sodium chloride, the wild type multiplied with a generation time of 3.5 h whereas the generation time of the mutant was 4.5 h. The difference between the strains was much higher at 1 M sodium chloride, as the mutant multiplied about 2-fold more slowly than the wild type and had a generation time of 34 h.

Conclusively, our results show that *M. smegmatis* is a halotolerant mycobacterial species that synthesizes ectoine and hydroxyectoine to protect itself against the deleterious effects of high salt concentrations. These mechanisms are of little significance when *M. smegmatis* is grown in isotonic growth media in the laboratory. In nature, however, osmoprotection likely makes a significant contribution to the ability of *M. smegmatis* to thrive in its habitat.

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