A Critical Process Controlled by MalT and OmpR Is Revealed through Synthetic Lethal∗

Sylvia A. Reimann and Alan J. Wolfe∗

Department of Microbiology and Immunology, Loyola University Chicago, 2160 S. First Ave., Bldg. 105, Maywood, Illinois 60153

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The death of cells harboring defects in two distinct pathways implicates these pathways in the control of an essential process. Here we report that cells lacking OmpR and harboring constitutively active MalT undergo premature death that involves increased expression of the outer membrane porin LamB.

To survive, a cell must coordinate its response to diverse stimuli simultaneously. These stimuli can originate in the extracellular environment, the envelope, or the cytosol. The coordination requires effective interaction of different, often apparently unrelated, pathways. A powerful tool to identify interactions between seemingly unrelated pathways is a genetic phenomenon called synthetic lethality (SL). SL is defined as the loss of viability that occurs from the combination of two mutations that separately do not cause death (14, 19, 22, 31). Here we used SL to demonstrate that the osmoregulator OmpR and MalT, the transcriptional regulator of the maltose uptake and metabolism system, interact to control a critical process. Loss of control by disruption of ompR in cells that also carry the malT(Con)(T949A) allele (initially termed SLompR) leads to cell death.

Construction and characterization of a conditional synthetic lethal mutant. While examining the effect of the small phosphorly donor acetyl phosphate on the function of the EnvZ-OmpR two-component signal transduction pathway, we attempted to construct an ackA ompR double mutant. We used P1 transduction (30) to introduce an ompR::Tn10 allele (from strain JB100) (Table 1) (2, 6) into an ackA null mutant (strain AJW1939) (18) and observed that approximately 50% of the resulting transductants (designated strain AJW2051) exhibited a set of unique phenotypes. During growth at 37°C on solid tryptone-based media (tryptone broth or LB), colonies became translucent and papillae arose. This behavior, contrasted with that of the parental strains and their wild-type (WT) parent (Fig. 1A). Similarly, following growth in liquid tryptone broth or LB, these transductants were unable to form viable colonies (Fig. 1B), a behavior that explains the loss of turbidity as the temperature increased.

The lack of OmpR causes cell death. To determine if disruption of ompR contributed to cell death, we employed pRC7, an unstable single-copy mini-F (mF) plasmid carrying ompR and lacZ under control of the isopropyl-β-d-thiogalactopyranoside (IPTG)-inducible lac promoter (3, 12). The mF plasmid lacks the natural F-factor stabilization systems; therefore, it is unstable and thus easily lost when no antibiotic selection is applied. If the vector carries an essential gene, cells have to maintain the plasmid even without antibiotic selection, and all colonies are blue. grea. If the plasmid does not carry an essential gene, it can be lost; thus, colonies with blue and white sectors and pure white colonies can form.

When cells of strain AJW2051 were transformed with mFompR, all surviving colonies were blue (Fig. 2A, middle panels). Similarly, in broth, single-copy mFompR (Fig. 2B) complemented the defect. Expression of WT OmpR from the multicopy pFR29 vector also complemented the growth defect. In contrast, expression of the mutant proteins OmpR(T83I) and OmpR(V203M) (20, 21) or of WT EnvZ did not complement the growth defect (data not shown). These results provide positive evidence that disruption of ompR contributes to cell death.

The lack of AckA does not cause cell death. We also tested if disruption of ackA contributed to cell death. In contrast to ompR, two distinct mFackA plasmids, one that included the native ackA ribosomal binding site (mFackA1) and one that included an optimized ribosomal binding site (mFackA2), failed to restore growth (Fig. 2A and B). Both plasmids exhibited AckA activity and complemented several ackA phenotypes, including mucoidy and poor motility (data not shown). Expression of WT ackA from a multicopy plasmid also failed to sustain growth of the mutant cells, and colonies transformed with ackA plasmids formed papillae (Fig. 2A, lower panels; data not shown). These observations indicated that disruption of ackA does not contribute to death. More importantly, they led us to hypothesize that strain AJW2051 carries an additional mutation [initially designated SLompR and later identified as

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Loyola University Chicago, 2160 S. First Ave., Bldg. 105, Maywood, IL 60153. Phone: (708) 216-5814. Fax: (708) 216-9574. E-mail: awolfe@luc.edu.

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were selected for both tetracycline and kanamycin resistance (and hence both the *ompR* and *canD* alleles) on M63 plates supplemented with 0.4% glucose and incubated at room temperature. Transductants were subsequently screened on LB plates at 37°C for translucency. Depending on the relative locations of *ompR*, *SL ompR*, and *canD*, the frequency of translucent colonies varied. Plotting the frequency of translucent offspring versus *canD* location in the genome generated a distinct pattern, which allowed us to map *SL ompR* to the *mal-rtc* locus (Fig. 3B).

Mapping also allowed us to make a statement about the nature of the *SL ompR* mutation. If *SL ompR* was a loss-of-function allele, then its transduction into the corresponding *canD* deletion mutant (i.e., the specific case where *SL ompR* is *canD*) would have resulted in 100% translucent colonies during mapping. However, Fig. 3B shows no such peak in the *mal-rtc* locus. On this basis, we concluded that *SL ompR* is unlikely to be a loss-of-function allele and thus tested the alternative possibility

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**TABLE 1.** Strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotypea</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AJW678</td>
<td>thi-1 thr-1(Am) leuB metF159(Am) rpsL136 lacX74</td>
<td>17</td>
</tr>
<tr>
<td>AJW1939</td>
<td>AJW678 ackA::Km</td>
<td>17</td>
</tr>
<tr>
<td>AJW2050</td>
<td>AJW678 <em>ompR::Tn10</em></td>
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<td>AJW2051</td>
<td>AJW678 <em>ompR::Km</em></td>
<td>This study</td>
</tr>
<tr>
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<td>AJW678 <em>ompR::Tn10</em></td>
<td>This study</td>
</tr>
<tr>
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<td>AJW678 <em>ompR::Tn10</em></td>
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<td>AJW678 malT con</td>
<td>This study</td>
</tr>
<tr>
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<td>AJW678 ackA::Km malT con</td>
<td>This study</td>
</tr>
<tr>
<td>JB100</td>
<td><em>ompR::Tn10</em> malT::Km-1</td>
<td>6</td>
</tr>
</tbody>
</table>

a All other alleles were derived from the KEIO collection (16).

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*malT*(Con)(T949A)] that is responsible for causing cell death in the absence of OmpR. To test this hypothesis, we introduced the *ompR::Tn10* allele from strain AJW2051 into cells of WT (ackA+) strain AJW678. Approximately 50% of the resulting transductants (strain AJW3098) died, and the dying colonies accumulated papillae. Taken together, these results led us to hypothesize that *SL ompR* is linked to the *ompR::Tn10* allele.

**Characterization of the *ompR SL ompR* mutant.** We characterized the *ompR* *SL ompR* double mutant (strain AJW3098) by comparing its phenotypes to those of an *ackA* *ompR* *SL ompR* triple mutant (strain AJW2051). The two mutants displayed the same translucent phenotype, and both of them accumulated papillae (Fig. 1A). The phenotype was universal; it could be observed in several different strain backgrounds (BW25113, MG1655, MC4100) and with different *ompR* null alleles (data not shown). Like the triple *ackA* *ompR* *SL ompR* mutant, the double *ompR* *SL ompR* mutant could be complemented with *mF-ompR* upon induction with IPTG. Subsequent depletion by back-dilution of the double mutant twice in fresh LB again led to a loss of turbidity, as observed with the complemented triple mutant (data not shown).

In contrast to the double mutant, the triple mutant grew more slowly, formed mucoid colonies, and migrated poorly in semisolid motility agar. These differences in growth, mucoidity, and motility are indicative of the *ackA* mutant allele (13). Furthermore, the triple mutant survived longer than the double mutant (data not shown) (Fig. 2C), most likely due to the slower growth attributed to the *ackA* mutation.

**Mapping of *SL ompR*.** To further map the location of *SL ompR*, we performed a series of tripartite crosses. We hypothesized that donation of the *ompR* *SL ompR* region to recipient strains using P1 transduction should yield a mixture of two different colony types. Type 1 colonies carrying both *ompR* and *SL ompR* should be nonviable and translucent when they are grown on or in tryptone-based media at 37°C. In contrast, type 2 colonies carrying *ompR* but not *SL ompR* should be viable and nontranslucent. We thus transferred the *ompR::Tn10* *SL ompR* region from the donor strain AJW2051 to recipient cells carrying null mutations in candidate genes (single-gene deletions, each designated *canD* and marked with kanamycin resistance) (1). *canD* genes were chosen to span the region that includes and flanks the *ompR* and *SL ompR* region (Fig. 3A). Transductants
that the SLompR mutation is a gain-of-function mutation. To test this hypothesis, we overexpressed each of the genes in the mal-rtc region in either the WT parent (strain AJW678) or the ompR mutant (strain AJW2050) with the expectation that, compared to the WT parent, the ompR mutant would display higher sensitivity to overexpression of at least one of these genes. We found that overexpression of gntT, rtcA, rtcB, rtcR, or malP had no significant effect on either the WT parent or the ompR mutant. In contrast, the ompR mutant was more sensitive than its WT parent to overexpression of malT or malQ (data not shown). At present, we cannot explain why an ompR mutant is more sensitive to malQ overexpression.

Subsequent sequencing revealed that the SLompR mutation is a point mutation located within the first one-third of the malT open reading frame. Replacement of thymine with adenine at position 949 (T949A) in the nucleotide sequence had led to replacement of a tryptophan residue (W) with an arginine residue (R) (Fig. 3C). We hypothesized that this amino acid change caused the production of a gain-of-function MalT [MalT(Con)] protein. Below, the SLompR mutation is referred to as malT(Con)(T949A) and the mutant protein is referred to as MalT(Con)(W317R).
We hypothesize that MalT(Con)(W317R) is a constitutively active protein. We tested (8), if malT(Con)(T949A) was responsible for SL in the ompR mutant background, then exposure to glucose should permit the ompR malT(Con)(T949A) mutant to survive in nonpermissive growth conditions. Indeed, when grown at 37°C in LB medium (either solid or liquid) supplemented with 0.4% glucose, cells of the ompR malT(Con)(T949A) mutant displayed WT-like growth (data not shown). This result is supported by our initial finding that the ompR malT(Con)(T949A) mutant survives on glucose-supplemented M63 minimal medium (data not shown) and supports our hypothesis that malT(Con)(T949A) encodes a constitutively active MalT protein.

Overexpression of MalY and Aes but not MalK permits survival of the ompR malT(Con)(T949A) mutant. Gain-of-function MalT [MalT(Con)] proteins can be inhibited by one or more of the following inhibitors: MalK, MalY, and Aes (16, 24, 28). MalK provides a feedback loop between maltose transport and MalT activation. Under conditions in which no maltose is transported into the cell, MalK directly interacts with MalT to inhibit activation by maltotriose (16). Like MalK, the inhibitors MalY and Aes also interfere with maltotriose binding (24, 28).

To determine if any of the inhibitors could inhibit MalT (Con)(W317R) and thus restore growth, we transformed the ompR malT(Con)(T949A) double mutant (strain AJW3098) with the empty pCA24N vector or with this vector carrying malK, malY, or aes (17). The resultant transformants were grown at 37°C on LB plates with increasing IPTG concentrations to induce expression of the inhibitors. We found that even uninduced expression of MalY or Aes permitted growth, likely due to leakiness of the T5-lac promoter (Fig. 4B). In contrast, expression of MalK did not permit survival even after induction with 0.2 mM IPTG (Fig. 4B and data not shown). The functionality of the plasmid was confirmed using outer membrane preparations to monitor the levels of LamB. As expected, expression of MalK in WT cells resulted in reduced LamB levels (data not shown). These experiments, combined with our outer membrane preparation, led us to conclude that malT(Con)(T949A) encodes a constitutively active MalT(Con) (W317R).

Deletion of lamB suppresses lethality. Since MalT activates transcription of all MalT regulon members, we hypothesized that increased expression of one or more of these proteins could cause lethality in an ompR mutant background. To identify the MalT regulon member that causes cell death in the absence of ompR, we introduced the ompR allele linked to the malT(Con)(T949A) mutation into several mal deletion mutants and screened for translucence. Of all the mal mutants tested (malEGFkmzs and lamB; malP and malQ were not tested), only the lamB ompR malT(Con)(T949A) and malK ompR malT(Con)(T949A) triple mutants produced 100% viable colonies. The viability of the malK ompR malT(Con) (T949A) triple mutant could be explained by a polar effect exerted by the malK allele on lamB. Indeed, removing the kanamycin resistance cassette using flp recombinase (10, 23) and thus generating a nonpolar deletion of malK produced nonviable colonies (data not shown). These results are consistent with the hypothesis that increased expression of LamB in the absence of OmpR contributes to death.

The observation that deletion of lamB permits survival of ompR malT(Con)(T949A) mutants may in part explain why the SL phenotype has not been reported before. Schlegel et al. (28) constructed several ompR malT(Con) double mutants in a strain background that includes the malB107 allele and thus lacks LamB (15).

SL is not specific to the malT(Con)(T949A) allele. Over the past four decades, several different malT(Con) alleles have been reported and studied (9, 28, 29). We asked if any of these alleles also cause a synthetic lethal phenotype in ompR mutations. The malT(Con)(T949A) mutant exhibits increased LamB levels. The transcription factor MalT positively regulates 10 genes organized in five operons (4, 5, 27). Constitutively active malT(Con) mutants are known to cause increased expression of all regulon members, including the outer membrane porin LamB (9, 11). To test if MalT(Con)(W317R) increases MalT regulon expression, we made outer membrane preparations and monitored LamB levels. We indeed found that the malT(Con)(T949A) (strain AJW33445) and ompR malT(Con)(T949A) (strain AJW3098) mutants exhibited increased LamB levels (Fig. 4A, lanes 3 and 4) compared to WT cells and the ompR mutant (Fig. 4A, lanes 1 and 2). This result provides further evidence that the SLompR mutation is located in malT.

We hypothesize that MalT(Con)(W317R) is a constitutively active protein.

SL of the ompR malT(Con)(T949A) mutant is subject to catabolite repression. Transcription of malT is regulated by catabolite repression; i.e., exposure to glucose represses malT transcription (7, 8). If malT(Con)(T949A) was responsible for SL in the ompR mutant background, then exposure to glucose should permit the ompR malT(Con)(T949A) mutant to survive in nonpermissive growth conditions. Indeed, when grown at 37°C in LB medium (either solid or liquid) supplemented with 0.4% glucose, cells of the ompR malT(Con)(T949A) mutant displayed WT-like growth (data not shown). This result is supported by our initial finding that the ompR malT(Con)(T9494A) mutant survives on glucose-supplemented M63 medium (data not shown) and supports our hypothesis that malT(Con)(T949A) encodes a constitutively active MalT protein.
tants. We cotransduced a representative selection of malT(Con) alleles, each linked to an ompR::Tn10 insertion (derived from strains AS42-46, AS48-50, AS54, and AS56) (28) into WT cells (strain AJW678). Transductants were selected as described above and screened for the translucent phenotype on LB plates at 37°C. We found that all of the malT(Con) alleles tested resulted in a translucent phenotype in an ompR mutant background (data not shown). Thus, we concluded that most, if not all, constitutively active MalT proteins cause death in mutants that lack OmpR.

Concluding remarks. Constitutively active MalT [in the form of MalT(Con)(W317R)] increases expression of LamB (9, 11). Surprisingly, such expression causes cell death in ompR mutants but not in cells that retain OmpR. There is no known overlap in the OmpR and MalT regulons; therefore, it is unlikely that the cause of death is redundancy of some essential function. Instead, we propose that cells coordinate the expression of the OmpR and MalT regulons to guard against the inappropriate insertion of LamB into the outer membrane. Efforts are under way to understand why LamB is detrimental to ompR mutants.

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