Posttranslational Maturation of the Invasion Acyl Carrier Protein of \textit{Salmonella enterica} Serovar Typhimurium Requires an Essential Phosphopantetheinyl Transferase of the Fatty Acid Biosynthesis Pathway

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\textit{Salmonella} pathogenicity island 1 (SPI-1) carries genes required for the formation of a type 3 secretion system, which is necessary for the invasion process of \textit{Salmonella}. Among the proteins encoded by SPI-1 is IacP, a homolog of acyl carrier proteins. Acyl carrier proteins are mainly involved in fatty acid biosynthesis, and they require posttranslational maturation by addition of a 4'-phosphopantetheine prosthetic group to be functional. In this study, we analyzed IacP maturation \textit{in vivo}. By performing matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) mass spectrometry analysis of intact purified proteins, we showed that IacP from \textit{Salmonella enterica} serovar Typhimurium was matured by addition of 4'-phosphopantetheine to the conserved serine 38 residue. Therefore, we searched for the phosphopantetheinyl transferases in charge of IacP maturation. A bacterial two-hybrid approach revealed that IacP interacted with AcpS, an enzyme normally required for the maturation of the canonical acyl carrier protein (ACP), which is involved in fatty acid biosynthesis. The creation of a conditional \textit{acps} mutant then demonstrated that AcpS was necessary for the maturation of IacP. However, although IacP was similar to ACP and matured by using the same enzyme, IacP could not replace the essential function of ACP in fatty acid synthesis. Hence, the demonstration that IacP is matured by AcpS establishes a cross-connection between virulence and fatty acid biosynthesis pathways.

\textit{Salmonella enterica} serovar Typhimurium is a facultative intracellular pathogen with a broad host spectrum that is a common cause of gastroenteritis in humans. Invasion of the nonphagocytic cells of the intestinal epithelium requires a type 3 secretion system (T3SS) encoded by \textit{Salmonella} pathogenicity island 1 (SPI-1). The SPI-1 T3SS allows translocation of bacterial effector proteins into the host cytoplasm, which trigger engulfment of the bacterial pathogen (1). Among the 35 genes harbored by SPI-1 is \textit{iacP}, encoding an invasion acyl carrier protein. Although SPI-1 has been identified and studied for decades (2, 3), little is known about IacP and its role in the invasion process.

Carrier proteins involved in diverse biosynthesis processes, such as fatty acid (FA), polyketide (PK), and nonribosomal peptide (NRP) synthesis, are small (80 to 100 residues), discrete proteins (type II) or modular domains of multienzymatic complexes (type I). These carrier proteins are first produced as apoproteins, and posttranslational modification, consisting of the attachment of a 4'-phosphopantetheine (4'-PP) prosthetic group on a conserved serine residue, occurs, generating holoproteins. The 4'-PP prosthetic group provides a terminal sulfhydryl group to tether intermediate metabolites (see Fig. S1 in the supplemental material). The transfer of 4'-PP from coenzyme A to the hydroxyl group of a conserved serine residue is catalyzed by phosphopantetheinyl transferases (PPTases). The best-studied member of this family of carrier proteins is the acyl carrier protein, ACP, involved in FA biosynthesis. In \textit{Escherichia coli}, ACP is an essential small acidic protein that shuttles the growing acyl chain from enzyme to enzyme during FA biosynthesis (4, 5). When acyl chains attain their final length, they are transferred by acyltransferases to phospholipid or lipid A precursors. Acyl-ACP is also used for acylation and maturation of the prohemolysin pro-HlyA (6, 7).

In \textit{E. coli}, maturation of ACP is carried out by AcpS, an essential PPTase due to its crucial function on ACP (8–10). AcpS was the first PPTase to be characterized, and homologs were not readily identified by sequence similarity. Refinement of the search motif finally revealed the existence of a PPTase superfamily, including AcpT and EntD in \textit{E. coli} (11). AcpT has been shown to partially compensate for the absence of AcpS (10, 12). However, if AcpT has another specific role in the cell, it remains mysterious. EntD is specifically involved in the biosynthesis of the siderophore molecule enterobactin and adds a 4'-PP group to the aryl and peptidyl carrier domains of EntB and EntF, respectively. Specificity between PPTases and their cognate substrates was revealed by the observation that EntD was efficient at transferring 4'-PP to apo-EntB and apo-EntF but not to apo-ACP \textit{in vitro}. Reciprocally, AcpS was efficient at transferring 4'-PP to apo-ACP but not to apo-EntB and apo-EntF \textit{in vitro} (11, 13, 14).

Although the canonical ACP involved in FA biosynthesis has been extensively studied, much less is known about the role and function of ACP-like proteins sometimes found in multiple copies in microorganisms (15). IacP and ACP are homologous and are both present in \textit{S. Typhimurium} (Fig. 1). A recent study has...
shown that IacP is a cytoplasmic protein necessary for invasion and full virulence of S. Typhimurium (16). The proposed explanation for this phenotype was that IacP might promote secretion and translocation of specific protein effectors: SopA, SopB, and SopD (16). It has also been reported that IacP might be involved in the regulation of flagellin expression (17). In the two studies, the conserved serine 38 residue was required for the observed roles of IacP, suggesting that maturation was occurring on IacP and was necessary for its activity. If true, this raises the question of the identity of the enzyme responsible for this maturation.

In this study, we demonstrated that IacP is indeed matured through phosphopantetheinylation on the conserved serine 38 residue, and we identified the essential PPTase AcpS, normally associated with FA biosynthesis, as responsible for IacP maturation.

MATERIALS AND METHODS

Bacterial strains and growth conditions. E. coli and S. Typhimurium strains used in this study are listed in Table 1. Bacteria were grown in 2YT or Luria-Bertani (LB) (Sigma-Aldrich). Ampicillin (100 μg/ml), kanamycin (25 or 50 μg/ml), spectinomycin (100 μg/ml), and chloramphenicol (30 μg/ml) were added when necessary. To trigger overexpression from the PPTase promoter and to control the overproduction of the recombinant PPTases, 400 ng/ml anhydrotetracycline was added at the start of the culture. Crude extracts were made 6 h later and analyzed by red Ponceau staining and Western blotting against the 6His tag.

For the PPTase activity complementation tests, constructions were made into the pASK-IBA37 plus vector (IBA), which is subsequently abbreviated pPTET. PPTase coding sequences were amplified using primers indicated in Table S1 in the supplemental material and were cloned into EcoRI/XhoI restriction sites of pT18Clink and pKT25link (21).

For the bacterial two-hybrid technique, pairs of proteins to be tested were fused to the C-terminal side of the two catalytic domains, T18 and T25, of adenylate cyclase. PCR products obtained using primers indicated in Table S1 in the supplemental material were inserted into EcoRI/XhoI restriction sites of pT18Clink and pKT25link (21).

To construct pKO3iacP, splicing by overlap extension PCR (22) using primers ebm133/ebm960 and ebm959/ebm675 was used to fuse the insert into pKO3 at the BamHI/SalI compatible restriction sites. Site-directed mutagenesis was performed on this plasmid using primers Ebm98 and Ebm99 and the QuickChange site-directed mutagenesis kit (Stratagene) to create pJV22. This construct was used as the template to produce a PCR product allowing lambda red-mediated recombination into the ΔiacP pKD46 strain in order to generate the IacP_S38T_TAP strain. To introduce a P_RAD upstream of the chromosomal coding sequence of acpS, a PCR product, including aadA7 (confering spectinomycin resistance), araC, and the P_RAD Promoter, was amplified from E. coli TG1 strain spec-arac (20) using primers Ebm904 and Ebm905.

For the bacterial two-hybrid technique, pairs of proteins to be tested were fused to the C-terminal side of the two catalytic domains, T18 and T25, of adenylate cyclase. PCR products obtained using primers indicated in Table S1 in the supplemental material were inserted into EcoRI/XhoI restriction sites of pT18Clink and pKT25link (21).

To construct pKO3iacP, splicing by overlap extension PCR (22) using primers ebm133/ebm960 and ebm959/ebm675 was used to fuse the E. coli acpP promoter to the ΔiacP gene. The corresponding DNA fragment was inserted into pKO3 (23) at the BamHI/Sall compatible restriction sites. E. coli and S. Typhimurium ACP have identical protein sequences, and previously made pT18-ACP and pKO3acpP were used (21, 31).

![Alignment of IacP and ACP proteins. Identical amino acid residues between the two proteins are indicated in the middle sequence, and similar residues are indicated by a plus sign. The conserved serine residue is in boldface, and the four α-helices present in the ACP structure are underlined.](https://example.com/alignment.png)
EGTA instead of 2 mM CaCl₂.

...calmodulin binding buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% NP-40) and were incubated on IgG-Sepharose beads (Pharmacia), and anti-His-horseradish peroxidase to detect His-tagged proteins (Santa Cruz Biotechnology). In parallel, a strain harboring a mutant allele of iacP could not be modified (strain JV56). Indeed, replacement of the equivalent conserved serine 36 of ACP by the similar amino acid residue threonine prevents the possibility of 4'-PP addition (29). The mass of purified IacP_CBP and IacP₁₃₈T_CBP was analyzed by MALDI-TOF mass spectrometry. Spectra showed a major peak at 14,589.6 Da for IacP_CBP bound to calmodulin (Fig. 2, black line), which, given an accuracy of ±2.8 Da for the mass measurement, corresponded to the expected theoretical mass [M + H]+ of 14,591.5 Da for IacP_CBP bound to 4'-PP, i.e., holo-IacP_CBP. In contrast, spectra showed a major peak at 14,265.6 Da for IacP₁₃₈T_CBP (Fig. 2, gray line), which corresponded to the expected theoretical mass [M + H]+ of 14,266.3 Da for apo-IacP₁₃₈T_CBP. In conclusion, those peaks could not represent IacP loaded with specific molecules attached to the 4'-PP group. Indeed, identical adducts were observed for the IacP₁₃₈T form, which is theoretically unable to load 4'-PP due to the presence of a serine residue at position 38. To confirm the identity of the adduct, an additional peak was observed at 14,589.6 Da for IacP_CBP bound to calmodulin (Fig. 2, black line), which corresponded to the expected theoretical mass [M + H]+ of 14,591.5 Da for holo-IacP_CBP.

**RESULTS AND DISCUSSION**

**Phosphopantetheinylation of IacP.** To assess if IacP is posttranslationally modified, mass spectrometry analysis was performed to determine the mass of the purified protein. For that purpose, a strain harboring a wild-type allele of iacP fused to the tandem affinity purification tag was engineered. The tagged version of iacP was placed at the original chromosomal locus, allowing physiological expression of the recombinant protein (strain JV48). The TAP tag sequence consists of two IgG binding domains of *Staphylococcus aureus* protein A (ProtA) and a calmodulin binding peptide (CBP) separated by a TEV protease cleavage (28). Two-step purification was realized based on these tags to finally recover IacP_CBP (see Fig. S2 in the supplemental material). In parallel, a strain harboring a mutant allele of iacP carrying the serine 38-to-threonine substitution fused to the TAP tag was also created in order to purify a mutated form of IacP that could not be modified (strain JV56). Indeed, replacement of the equivalent conserved serine 36 of ACP by the similar amino acid residue threonine prevents the possibility of 4'-PP addition (29). The mass of purified IacP_CBP and IacP₁₃₈T_CBP was analyzed by MALDI-TOF mass spectrometry. Spectra showed a major peak at 14,589.6 Da for IacP_CBP (Fig. 2, black line), which, given an accuracy of ±2.8 Da for the mass measurement, corresponded to the expected theoretical mass [M + H]+ of 14,591.5 Da for IacP₁₃₈T_CBP bound to 4'-PP, i.e., holo-IacP₁₃₈T_CBP. In contrast, spectra showed a major peak at 14,265.6 Da for IacP₁₃₈T_CBP (Fig. 2, gray line), which corresponded to the expected theoretical mass [M + H]+ of 14,266.3 Da for the unmodified IacP₁₃₈T_CBP. In conclusion, those results showed that 4'-PP was attached to IacP_CBP but not to IacP₁₃₈T_CBP, indicating that phosphopantetheinylation was occurring on serine 38 of IacP. Mass spectra showed additional peaks in a window of +10 to +500 Da larger than the major peaks (Fig. 2). However, those peaks could not represent IacP loaded with specific molecules attached to the 4'-PP group. Indeed, identical adducts were observed for the IacP₁₃₈T form, which is theoretically unable to load intermediate metabolites. The main additional peaks corresponding to adducts of +76 and +96 Da might result from technical issues and might correspond to molecules of β-mercaptoethanol and a trifluoroacetyl ion, respectively, that are present during the experiment and might be bound to the analyzed proteins.
Interaction between IacP and the PPTase AcpS. Attachment of the 4'-PP prosthetic group onto carrier proteins is carried out by PPTases. There is no gene coding for a putative PPTase in the genomic environment of iacP, suggesting that IacP does not have an exclusively dedicated PPTase. Based on what was published about E. coli PPTases, we found the same three PPTase genes, acpS, acpT, and entD, annotated in the genome of S. Typhimurium (http://www.genome.jp/kegg/). Each of the three PPTase protein sequences were used to blast the S. Typhimurium protein library to search for additional putative PPTases. We identified the YieE protein of unknown function, most similar to AcpT, as a fourth putative PPTase (see Fig. S3 in the supplemental material).

A bacterial two-hybrid approach was chosen to test protein-protein interactions between IacP and each of the four PPTases (25, 30). As a proof of concept, we first tested the interaction between ACP and AcpS, which is the PPTase-maturing ACP (8–10). However, no interaction was detected between ACP and AcpS or between ACP and the other PPTases (data not shown). We reasoned that the bacterial two-hybrid test was not sensitive enough to detect transient interaction between an enzyme and its substrate that would be quickly modified. We hypothesized that by mutating the conserved serine residue where modification occurs, the reaction of the enzyme on its substrate would be prevented but the recognition between the two proteins would be maintained, and the interaction between the enzyme and the inactive substrate would even been prolonged. Therefore, bacterial two-hybrid tests were repeated using the ACP<sub>P<sub>S<sub>36</sub>T</sub> version, which is an inactive substrate of AcpS (10). Under these conditions, a clear interaction was revealed between ACP<sub>P<sub>S<sub>36</sub>T</sub> and AcpS but not between ACP<sub>S<sub>S<sub>57</sub>T</sub> and the other PPTases (Fig. 3). Next, interactions between IacP, IacP<sub>S<sub>S<sub>38</sub>T</sub> and PPTases were tested. Only the coexpression of pT25-IacP<sub>S<sub>S<sub>38</sub>T</sub> and pT18-AcpS gave significant β-galactosidase activity (Fig. 3). Interaction could not be detected between IacP<sub>S<sub>S<sub>57</sub>T</sub> and the other PPTases (data not shown). Overall, these results showed an interaction between IacP and AcpS but not between IacP and the other PPTases, suggesting that AcpS is the PPTase responsible for the posttranslational modification occurring on IacP.

**TABLE 2 Capacity of the conditional acpS mutants to grow as isolated colonies**

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<th>Strain</th>
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<tr>
<td></td>
<td>LB agar</td>
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<tr>
<td></td>
<td>(arabinose 0.2%)</td>
</tr>
<tr>
<td>iacP_TAP</td>
<td>+</td>
</tr>
<tr>
<td>P&lt;sub&gt;BAD&lt;/sub&gt;acpS IacP_TAP</td>
<td>+</td>
</tr>
<tr>
<td>P&lt;sub&gt;BAD&lt;/sub&gt;acpS IacP_TAP pP&lt;sub&gt;TET&lt;/sub&gt;</td>
<td>+</td>
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<td>P&lt;sub&gt;BAD&lt;/sub&gt;acpS IacP_TAP pP&lt;sub&gt;TET&lt;/sub&gt;acpS</td>
<td>+</td>
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<tr>
<td>P&lt;sub&gt;BAD&lt;/sub&gt;acpS IacP_TAP pP&lt;sub&gt;TET&lt;/sub&gt;entD</td>
<td>+</td>
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<tr>
<td>P&lt;sub&gt;BAD&lt;/sub&gt;acpS IacP_TAP pP&lt;sub&gt;TET&lt;/sub&gt;yieE</td>
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* Strains were isolated on LB agar media with or without 0.2% arabinose and incubated overnight at 37°C.

The PPTase AcpS is essential in *Salmonella*. In *E. coli*, acpS is an essential gene, suggesting that this is also the case in *Salmonella*. Therefore, deletion of the *acpS* gene, to further demonstrate the role of AcpS in the phosphopantetheinylation process of IacP, may be inappropriate. Instead, we chose to insert a P<sub>BAD</sub> promoter at the chromosomal locus upstream of *acpS*, using the REXBAD cassette described by Roux et al. (20). Under the control of P<sub>BAD</sub>, the expression of acpS could be induced by addition of arabinose into the culture medium. This genetic manipulation was realized in the strain expressing IacP_TAP to create P<sub>BAD</sub> acpS IacP_TAP (strain JV68).

Growth of the P<sub>BAD</sub> acpS IacP_TAP strain (JV68) was tested on agar LB plates. The P<sub>BAD</sub> acpS IacP_TAP strain was only able to grow in the presence of arabinose (inducing conditions for P<sub>BAD</sub>). On the other hand, the parental control strain IacP_TAP (JV48) was able to grow on LB media with or without arabinose (Table 2; also see Fig. S4A in the supplemental material). Growth of the two strains was also monitored in liquid LB media with arabinose or glucose (inducing and repressing conditions for P<sub>BAD</sub>, respectively) (Fig. 4). From various growth experiments, we observed that about eight generations were necessary to deplete AcpS and to observe growth defects of the conditional acpS mutant. Hence, Fig. 4 shows growth curves of cultures that were first grown for about 5 generations before being back diluted to an OD<sub>600</sub> of 0.05, and then the optical density was monitored. Those results showed that AcpS is essential in *Salmonella*.

**Complementation of the lethal effect of AcpS depletion.** Complementation experiments were performed to rescue growth of the conditional acpS mutant. Each PPTase-encoding gene was
cloned into pPTET. This vector allows expression of recombinant protein under the control of the TET promoter, which is inducible by addition of anhydrotetracycline. The strain P_BAD acpS lacP_TAP was transformed by each of the pPTET PPTase constructions and tested for the capacity to grow on an agar LB plate with or without arabinose. Only P_BAD acpS lacP_TACP_TAP grew normally in the absence of arabinose (Table 2; see also Fig. S4B in the supplemental material). Growth complementation conferred by the presence of pPTETacpS was effective even without addition of anhydrotetracycline to the LB plate, indicating that the amount of AcpS produced by leakage of the PTET promoter was sufficient for growth complementation. Interestingly, P_BAD acpS lacP_TACP_TAP, carrying pPTETentD, was able to grow in the absence of arabinose, but small colonies were produced (Table 2; also see Fig. S4B). P_BAD acpS lacP_TACP_TAP harboring pPTETacpT or pPTETyieE did not show any growth rescue. The overproduction of the recombinant proteins by addition of anhydrotetracycline in LB agar did not change the overall growth pattern of each strain (data not shown). A control production test, however, indicated that PPTases were indeed produced upon addition of anhydrotetracycline to liquid cultures (data not shown). As acpT has been proposed to function as a backup for acpS in E. coli (10, 12), we were surprised to see no complementation using pPTETacpT. However, we cannot exclude that production of a recombinant PPTase fused to a N-terminal 6His tag (present in pPTET) is detrimental to AcpT activity. These results showed that the growth defect due to depletion of AcpS could be fully rescued by expression of the corresponding gene in trans and partially rescued by overexpression of entD.

AcP is matured by the PPTase AcP. Maturation of AcP and detection of an interaction between AcP and AcpS by the bacterial two-hybrid method suggested that AcpS was the enzyme responsible for AcP maturation. To test this hypothesis, we studied the effect of the depletion of AcpS on the posttranslational modification of AcP. IacP_CBP was purified from strain P_BAD acpS lacP_TACP_TAP (JV68) grown with (plus arabinose) or without (plus glucose) AcpS production. MALDI-TOF mass spectrometry analysis of intact proteins then was performed. The spectrum of IacP_CBP, purified while AcpS was produced (with arabinose), showed a major peak at 14,590 ± 2.8 Da that corresponded to IacP_CBP bound to 4’-PP, for which the theoretical mass [M + H]+ was 14,591.5 Da (Fig. 5A, black spectrum). However, when IacP_CBP was purified while AcpS was depleted (with glucose), the major peak for IacP_CBP shifted from 14,590 ± 2.8 Da to 14,250.4 ± 2.8 Da (Fig. 5A, gray spectrum). The peak at 14,250.4 ± 2.8 Da corresponded to unmodified IacP_CBP, for which the theoretical mass [M + H]+ was 14,252.2 Da. This indicated a maturation defect of IacP in the absence of AcpS.

To determine if a PPTase other than AcpS could promote maturation of IacP, protein crude extracts were prepared from P_BAD acpS lacP_TACP_TAP grown under conditions where expression of chromosomal acpS was off and while one of the 4 PPTases was present in trans under the control of P_TET. IacP_TACP_TAP was then detected by Western blotting (Fig. 5B). Previous experiments with the control strains JV48 and JV56, producing IacP_TACP and IacP_SSET_TACP, respectively, had shown that IacP_TACP_TAP typically ran on SDS-PAGE as a doublet, with the upper band being more intense (Fig. 5B, lane 1), while IacP_SSET_TACP typically ran on SDS-PAGE as a single lower band (Fig. 5B, lane 2). We took advantage of this mobility pattern on SDS-PAGE to conveniently analyze the maturation status of IacP_TACP from the P_BAD acpS lacP_TACP strain. First, we observed that IacP_TACP ran predominantly as the apo form when chromosomal expression of acpS was off (absence of arabinose) (Fig. 5B, lane 3) and ran predominantly as the holo form when chromosomal expression of acpS was on (presence of arabinose) (Fig. 5B, lane 4), which is in agreement with the mass spectrometry results (Fig. 5A). We then analyzed IacP_TACP from P_BAD acpS lacP_TACP carrying one of the pPTET PPTase plasmids, while chromosomal expression of acpS was off. IacP_TACP showed the characteristic pattern of holo IacP only when P_BAD acpS lacP_TACP carried pPTETacpS (Fig. 5B, compare lane 6 to lanes 5, 7, 8, and 9).

The presence of acpT or yieE in trans seemed detrimental for IacP production, since less protein was detected by Western blotting. This was possibly linked to disturbed growth. Indeed, we noticed repeatedly that expression of acpT, and especially yieE, was detrimental to growth (data not shown). In conclusion, only AcpS seemed to promote IacP maturation.
tions were introduced into *E. coli* MG1655. The capacity to transduce the Δ*acpP*:Kan’ allele (from EB689) into MG1655 harboring pKO3, pKO3*acpP*, or pKO3*iacP* was assayed on LB agar plates supplemented with kanamycin. Clones were obtained only when the *E. coli* strain harbored pKO3*acpP* (see Fig. S5B). Overall, two different tests indicated that IacP could not replace the essential functions of ACP.

**Conclusions.** Whereas *E. coli* K-12 harbors only one gene encoding ACP, bacteria with more complex metabolisms or lifestyles often possess multiple ACP elements. This study shows for the first time the maturation process of an acyl carrier protein associated with the invasion machinery of a bacterial pathogen. Using a conditional *acpS* mutant, we were able to show in vivo the unambiguous involvement of AcpS in the maturation process of IacP. The use of an essential function as the one performed by AcpS may ensure that the bacterial pathogen maintains the capacity to mature the virulence-associated protein IacP. As mentioned earlier, IacP has been proposed to promote the secretion of the protein effectors SopA, SopB, and SopD (16) and to influence the Hin DNA invertase-mediated flagellar phase variation (17). However, in light of what is known about the mode of action of ACPs, it is unclear at this stage how IacP would regulate these events. Now that we have shown that phosphopantetheinylation occurs on IacP thanks to AcpS, the existence of further connections between FA biosynthesis and virulence pathways has to be explored. The next challenge will be to demonstrate that IacP is loaded with FA and to determine the nature and final destination of the FA carried by IacP.

**TABLE 3** Capacity of ACP*acps* transformants to form colonies at nonpermissive temperature

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<tr>
<td>ACP<em>acps</em> pT18</td>
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<tr>
<td>ACP<em>acps</em> pT18-ACP</td>
<td>+</td>
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<tr>
<td>ACP<em>acps</em> pT18-IacP</td>
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 discovered ACPs.

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<tr>
<td>ACP<em>acps</em> pT18-IacP</td>
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*The *E. coli* ACP*acps* strain was transformed with the indicated plasmids, spread on selective LB agar plates, and incubated for 3 days at 30 or 42°C.*

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ful discussions.

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