Indole inhibits bacterial quorum sensing signal transmission by interfering with quorum sensing regulator folding

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Quorum sensing (QS)-dependent biofilm formation and motility were controlled by AqsR in *Acinetobacter oleivorans* DR1. QS-controlled phenotypes appeared to be inhibited by indole and the aqsR mutant had the same phenotypes. We demonstrated that the turnover rate of AqsR became more rapid without the N-acylhomoserine lactone (AHL) signal, and that indole could increase the expression of many protease and chaperone proteins. The addition of exogenous indole decreased the expression of two AqsR-targeted genes: AOLE_03905 (putative surface adhesion protein) and AOLE_11355 (l-asparaginase). The overexpression of AqsR in *Escherichia coli* was impossible with the indole treatment. Surprisingly, our[^35S]methionine pulse-labelling data demonstrated that the stability and folding of AqsR protein decreased in the presence of indole without changing aqsR mRNA expression in *E. coli*. Interestingly, indole resulted in a loss of TraR-dependent traG expression in an *Agrobacterium tumefaciens* indicator strain. However, when indole was added after incubation with exogenous AHL, indole could not inhibit the TraR-dependent expression of the traG promoter. This indicated that AHL-bound TraR could be protective against indole, but TraR without AHL could not be active in the presence of indole. Here, we provided evidence for the first time showing that the indole effect on QS-controlled bacterial phenotypes is due to inhibited QS regulator folding and not a reduced QS signal.

**INTRODUCTION**

Quorum sensing (QS) is a cell-density-dependent form of bacterial communication (Fuqua et al., 1994; Williams, 2007). QS is mediated by small diffusible signal molecules that are produced by many Gram-negative bacteria during the stationary phase of growth. LuxI homologues synthesize a variety of N-acylhomoserine lactones (AHLs) as QS signals (Williams, 2007). LuxR-type regulators bind to their cognate QS signals, which changes the expression of many genes whose products control various physiological and morphological traits, such as biofilm formation, motility, virulence and antibiotic production (Zhu et al., 2002; Hentzer et al., 2003; Stanley & Lazazzera, 2004; Rader et al., 2007; Waters et al., 2008; Weiss et al., 2008; Duerkop et al., 2009). LuxR-type regulators appear to require their cognate signal for their folding and protease resistance (Zhu & Winans, 2001; Vannini et al., 2002; Zhang et al., 2002). Complexes between the LuxR regulators and AHLs attain their correctly folded configuration, whereas LuxR synthesized in the absence of AHLs is rapidly degraded (Zhu & Winans, 2001; Costa et al., 2012).

Recent studies demonstrated that *Acinetobacter* species also have a QS system and that their biofilm formation ability was controlled by QS signals (Sarkar & Chakraborty, 2008; Tomaras et al., 2008; Kang & Park, 2010a). Pathogenic *Acinetobacter baumannii* forms biofilms on a surface using pili, extracellular polymeric substances and surface adhesion proteins, which play important roles in infection and antibiotic resistance (Tomaras et al., 2008; Gaddy & Actis, 2009). These critical factors in biofilm formation were shown to be controlled by QS systems (Gaddy & Actis, 2009). QS systems in non-pathogenic *Acinetobacter* species have been poorly explored. Under environmental conditions, non-pathogenic *Acinetobacter* can also form biofilms on both abiotic and biotic surfaces (Rosenberg et al., 1982; Gohl et al., 2006; González et al., 2009). It has been reported that biofilm formation appears to be very important for degrading hydrocarbons at the hydrocarbon/water interface (Kang & Park, 2010a; Baldi et al., 1999).

Previously, we isolated diesel-degrading *Acinetobacter oleivorans* DRI from a rice paddy (Jung et al., 2010). Genomic data and experimental data supported the finding that DRI cells have a QS system consisting of a LuxR-type regulator and a LuxI-type AHL synthase protein, designated AqsR and Aqsl, respectively (Kang & Park, 2010a). We demonstrated

**Abbreviations:** AHL, N-acylhomoserine lactone; qRT, quantitative reverse transcriptase; QS, quorum sensing.
that QS signals play an important role in hexadecane biodegradation and biofilm formation. The QS signal in DR1 was decreased in the Pseudomonas sp. AS1 and DR1 co-culture system because of the quorum quenching activity in the AS1 strain (Seo et al., 2012). These effects alter biofilm formation physiologically because of the absence of the QS signal. The loss of the QS signal-producing activity had an effect on protein expression in DR1 (Kang & Park, 2010a, b). The QS regulator AqsR functions as an important regulator and is associated with several phenotypes, such as hexadecane utilization, biofilm formation and sensitivity to cumene hydroperoxide, which were confirmed using an aqsR mutant and RNA sequencing analysis. Our electrophoretic mobility shift assays revealed that AqsR directly bound to the promoter regions of AOLE_03905 (SapA, putative surface adhesion protein) and AOLE_11355 (l-asparaginase) (Kim & Park, 2013).

Many bacteria, when they live in the natural environment or human intestines, form various metabolites and secrete them from the cells (Helling et al., 2002). Escherichia coli can excrete considerable amounts of indole and acetate in the stationary phase (Helling et al., 2002; Kobayashi et al., 2006). Indole has received great attention because of its extensive effects on various biological functions in the bacterial population, such as biofilm formation, antibiotic resistance, and virulence. Indole and acetate can act as a proton ionophore and inhibit cell division because of reduced electrochemical potential when indole passes through the membrane (Chimerel et al., 2012). Interestingly, we demonstrated recently that indole caused toxicity to Pseudomonas putida and interfered with protein folding (Kim et al., 2013). Highly expressed genes coding for proteases, molecular chaperones and TCA cycle enzymes in the presence of indole might play crucial roles in indole-induced stress conditions. Indole has been reported to act as an antivirulence compound against E. coli, Pseudomonas aeruginosa and Staphylococcus aureus (Lee et al., 2007, 2009, 2013; Chu et al., 2012). Indole reduced virulence factors by modulating the expression of virulence and regulatory genes, which were reportedly regulated by the QS system in P. aeruginosa (Lee et al., 2009). Here, we provide evidence that indole might reduce QS regulator folding, leading to the differential expression of many QS-controlled genes.

**METHODS**

**Bacterial strains and culture conditions.** The bacterial strains used in this study are shown in Table 1. A. oleivorans DR1 was grown at 30 °C in nutrient broth with aeration by shaking. E. coli was grown at 37 °C in LB medium. When required, antibiotics were added at the following concentrations: 100 μg ml⁻¹ ampicillin, 50 μg ml⁻¹ kanamycin and 50 μg ml⁻¹ gentamicin. Growth was monitored by measuring the OD₆₀₀ of the cultures using a biophotometer (Eppendorf). The complete genome sequence of strain DR1 can be found in GenBank (accession no. CP002080). Strain DR1 was deposited in the Korea Collection for Type Cultures (KCTC 23045) and the Japan Collection of Microorganisms (ICM 16667).

**Detection of the QS signal and β-galactosidase assays.** AHLs of the DR1 were detected using the Agrobacterium tumefaciens C58 (pZLR4) indicator strain (Cha et al., 1998). The supernatant of the cell culture was collected via centrifugation (12 000 g, 30 min) at 4 °C, and the pellets were removed. The cell-free supernatant was prepared via filter sterilization with 0.22 μm pore filters. Detection of QS signals and the inhibition of TraR folding were determined by a β-galactosidase assay. The cell-free supernatants to detect the QS signals were collected as described above and added individually to medium in which the exponentially growing (OD₆₀₀ ~0.3) indicator strain was inoculated. To determine the inhibition of A. tumefaciens TraR-controlled gene induction by indole, indicator cells were grown overnight in LB medium at 30 °C, diluted 100-fold in fresh M9 medium containing 1% glucose and 0.3% Casamino acids (Kang & Park, 2010b), and 5 μM AHL (N-3-oxooctanoyl-L-homoserine lactone; Sigma) and/or 1 mM indole were added. The cultures were incubated for 24 h until they reached the stationary phase. The presence of AHL or the inhibition of TraR-controlled genes was quantified by measuring β-galactosidase activity using o-nitrophenyl-β-D-galactopyranoside as a substrate (Miller, 1992).

**Abiotic biofilm formation and motility assay.** The biofilm formation assay was conducted as described previously (Kang & Park, 2010a; Seo et al., 2012). Bacterial cells were inoculated into nutrient broth and incubated for 24 h at 30 °C. Cells were washed twice in PBS, inoculated at 10⁶ cells in nutrient medium and grown in 96-well polystyrene microtitre plates (Costar) for 24 h at 30 °C under static conditions. Biofilm formation was measured by staining the attached cells with crystal violet. After staining, the attached cells were resuspended in ethanol and the absorbance was measured at 595 nm. A microplate spectrophotometer (PowerWave XS; Bio-Tek) was used to measure the OD.

For the swimming motility assay, a plate containing ~20 ml of semi-solid nutrient medium (0.2% agar) was spotted with exponentially grown bacteria. The plates were incubated for 24 h at 30 °C.

**Gene expression analysis by Northern blotting.** Total RNA was isolated using an RNaseasy kit (Qiagen) according to the manufacturer’s instructions. Northern blot analysis was then performed as described previously (Kang et al., 2006; Lee et al., 2006). RNA concentrations were estimated by the absorbance at 260 nm. Samples of total RNA (5 μg) were loaded on denaturing agarose gels containing 0.25 M formaldehyde, separated, and then stained with ethidium bromide to visualize 23S and 16S rRNA. The fractionated RNA was transferred to nylon membranes (Schleicher & Schuell) using a Turboblotter (Schleicher & Schuell). The amount of mRNA [AOLE_03905 (sapA) and AOLE_11355] was determined by hybridizing the membrane with a specific 32P-labelled probe (Takara) that was prepared by PCR amplification with the respective primer pairs as indicated in Table 1. Autoradiography was conducted using an IP plate (FujiFilm) and a Multiplex Bio-Imaging System (FujiFilm).

**Measurements of AqsR turnover in E. coli.** The measurement of AqsR turnover was conducted as previously described (Zhu & Winans, 2001). To radiolabel AqsR protein, E. coli strain BL21 (AqsR) or BL21 (AqsR, AqI) (Kim & Park, 2013) was cultured at 30 °C in LB
medium. Cells were grown exponentially (OD₆₀₀ ~0.4) at 37 °C. At OD₆₀₀ ~0.4, the culture was treated with IPTG to a final concentration of 0.5 mM. To demonstrate the effect of indole on AqsR protein folding, 1 mM indole was simultaneously added with IPTG. After 30 min, rifampicin was added to a final concentration of 0.5 mM. To demonstrate the effect of indole on the IPTG. After 30 min, rifampicin was added to a final concentration of 0.5 mM. To demonstrate the effect of indole on

In our previous studies, we demonstrated that QS plays an important role in biofilm formation, motility and hexadecane biodegradation in *A. oleivorans*, and the QS signal binding to the QS regulator, AqsR, might be an N-(3-oxododecanoyl)-homoserine lactone (Kang & Park, 2010a). We also demonstrated that AqsR functions as a crucial regulator in biofilm formation with direct regulation of genes, including the surface adhesion protein (Kim & Park, 2013). Interestingly, indole inhibited biofilm formation of *A. oleivorans* (Fig. 1a). The *aqsR* mutant had reduced swimming motility (Fig. 1b). Indole reduced the swimming motility of *A. oleivorans* (Fig. 1b). The pellicle formation in liquid cultures appeared to be inhibited by indole and the *aqsR* mutant had the same phenotype (data not shown). Our data demonstrated that WT cells that were treated with indole had the same phenotypes (reduced biofilm formation, loss of swimming motility and inhibited pellicle formation) as the negative control. No phenotypic changes from the ethanol were observed. The amount of signal that was secreted by *A. oleivorans* aqsR mutant. As the indole was

**Table 1. Bacterial stains and primers used in this study**

<table>
<thead>
<tr>
<th>Strain or primer</th>
<th>Description or sequence (5’→3’)</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Acinetobacter oleivorans</em> DR1</td>
<td>WT, diesel oil degrader</td>
<td>Jung et al. (2010)</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>pET-28a (+) - <em>aqsR</em> into <em>E. coli</em> BL21(DE3)</td>
<td>Kim &amp; Park (2013)</td>
</tr>
<tr>
<td><em>Agrobacterium tumefaciens</em> C58 (pZLR4)</td>
<td>pBBR1MCS4- <em>aql</em> into <em>E. coli</em> BL21(DE3) [pET-28a (+) - <em>aqsR</em>]</td>
<td>Kim &amp; Park (2013)</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>AHL indicator</td>
<td>Cha et al. (1998)</td>
</tr>
<tr>
<td><em>Chromobacterium violaceum</em></td>
<td>ATTC 12472 strain, WT</td>
<td>Laboratory stock</td>
</tr>
</tbody>
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**Quantitative reverse transcriptase (qRT)-PCR.** Total RNA was isolated from 5 ml of cells in the exponential (OD₆₀₀ ~0.4) and stationary (OD₆₀₀ ~2.0) phases using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. cDNA was synthesized from 1 μg of RNA with primers of three genes (groES, lon and hsp90) (Table 1) and used as templates for qRT-PCR. The PCR mixture contained 12.5 μl of iQ SYBR Green Supermix (Bio-Rad), 1 μl of each primer (0.5 μM) and 2 μl of cDNA in a total volume of 25 μl. The PCR conditions were 95 °C for 3 min, followed by 40 cycles of 45 s at 95 °C, 45 s at 60 °C and 45 s at 72 °C. To normalize the expression of each gene, the expression level of 16S rDNA was quantified with primers that were used previously (Watanabe et al., 2001). The quantification results were calculated from triplicate experiments.

**RESULTS**

**Inhibition of QS-controlled phenotypes by indole**

In our previous studies, we demonstrated that QS plays an important role in biofilm formation, motility and hexadecane biodegradation in *A. oleivorans*, and the QS signal binding to the QS regulator, AqsR, might be an N-(3-oxododecanoyl)-homoserine lactone (Kang & Park, 2010a). We also demonstrated that AqsR functions as a crucial regulator in biofilm formation with direct regulation of genes, including the surface adhesion protein (Kim & Park, 2013). Interestingly, indole inhibited biofilm formation of *A. oleivorans* (Fig. 1a). The *aqsR* mutant had reduced swimming motility (Fig. 1b). Indole reduced the swimming motility of *A. oleivorans* (Fig. 1b). The pellicle formation in liquid cultures appeared to be inhibited by indole and the *aqsR* mutant had the same phenotype (data not shown). Our data demonstrated that WT cells that were treated with indole had the same phenotypes (reduced biofilm formation, loss of swimming motility and inhibited pellicle formation) as the negative control. No phenotypic changes from the ethanol were observed. The amount of signal that was secreted by *A. oleivorans* differed in the presence of different indole concentrations (Fig. 1c). The reduced QS signal production was not because of growth defects in the presence of indole (Fig. 1d). This phenomenon might be due to the fact that the production of some QS signals is reported to be regulated positively by QS regulators (Lazdunski et al., 2010).
Our data also suggested that the QS signal production of *A. oleivorans* was regulated positively by AqsR (Fig. 1e). The aqsR mutant produced less of the QS signal.

**Decreased expression of the AqsR target genes with indole**

Our previous data showed that AOLE_03905 (SapA, putative surface adhesion protein) and AOLE_11355 (L-asparaginase) are controlled by AqsR in the stationary phase (Kim & Park, 2013). Our Northern blot analysis showed that indole decreased the expression of *sapA* and AOLE_11355 genes (Fig. 2a, b). We added indole at different time points to check how long indole affects and when indole reduces the expression of the AqsR-targeted genes during growth (Fig. 2c). The cell incubation continued until cells reached the stationary phase (OD$_{600}$ ~2.0), when mRNA was extracted. Our Northern blot analysis showed that *sapA* and AOLE_11355 genes were induced in the stationary-phase cells (Fig. 2d, e). However, indole resulted in the loss of *sapA* and AOLE_11355 induction (Fig. 2d, e). Our data showed that indole inhibited QS-controlled gene expression in *A. oleivorans*.

**Inhibition of AqsR protein folding by indole**

We demonstrated previously that indole induced many proteases and chaperone proteins, probably because indole affected protein stability and folding, which was demonstrated by an *in vitro* protein folding assay with indole (Kim *et al.*, 2013). As the QS regulator was reported to be unstable without the cognate AHL signal (Zhu & Winans, 2001; Vannini *et al.*, 2002; Zhang *et al.*, 2002), we measured the half-life of AqsR protein in the presence and absence of the AHL signal (Fig. 3a). When AqsR alone was overexpressed in *E. coli*, rapid degradation of AqsR was observed using the $[^{35}\text{S}]$methionine pulse-labelling assay (Fig. 3a). This rapid degradation was not observed in the presence of the cognate QS signal, which could be possible with the coexpression of AqsI (Fig. 3a). The half-lives of AqsR alone and AqsR with AHL were 59.8 and 232.4 min, respectively, under the conditions that we used (Fig. 3b).
These data confirmed that the folding and stability of AqsR require its cognate AHL, which is consistent with the findings of other reports (Zhu & Winans, 2001; Vannini et al., 2002; Zhang et al., 2002; Costa et al., 2012). We speculated that AqsR could be unstable in the presence of indole, which causes QS-controlled phenotypic losses and inhibits the expression of genes that are regulated by AqsR. To test whether indole could affect AqsR protein expression and degradation, we used the AqsR overexpression strain and [35S]methionine pulse labelling. To ensure that IPTG transport into cells was not inhibited by indole, a much higher concentration of IPTG (2 mM) than the usual induction concentration (0.25–0.5 mM) was also tested. Surprisingly, AqsR was not overexpressed in the presence of indole (Fig. 3c). Our Northern blot assay confirmed that aqsR mRNA induction was not inhibited by indole (Fig. 3d). Therefore, indole appeared to affect AqsR folding, but not its gene expression, which could lead to the degradation of AqsR.

**Inhibition of A. tumefaciens TraR-controlled gene induction by indole**

Our AqsR data suggested strongly that the QS regulator could not be folded properly in the presence of indole. This phenomenon might not be plausible if the QS regulator is already folded with its cognate QS signal, which stabilizes the QS regulator as illustrated in Fig. 3(a). We tested this hypothesis using the A. tumefaciens TraR system, which harbours TraR and the traG promoter–lacZ reporter fusion
The chaperone proteins GroEL/ES play an important role in TraR folding and increase the expression of TraR-dependent genes (Chai & Winans, 2009). When exogenous AHL was added to the A. tumefaciens indicator strain, the QS-controlled induction was observed clearly (Fig. 4). However, this induction was reduced in the presence of indole. Interestingly, indole that was added after incubation with AHL did not inhibit the TraR-dependent expression of the traG promoter. When AHL was added after incubation with indole, gene expression was not inhibited. These data suggested strongly that AHL-bound TraR could be protective against indole, but TraR without AHL could not fold properly in the presence of indole. Thus, our data provide evidence that indole might not affect TraR-controlled gene expression when AHL binds to TraR.

**Effect of indole on the expression of chaperone and protease in A. oleivorans**

Many genes involved in chaperone and protease functions were induced by indole in Pseudomonas putida (Kim et al., 2013). Several chaperone and protease proteins (Lon, GroES and Hsp90) appeared to be upregulated highly in A. oleivorans at both the exponential and stationary phases with the addition of indole (Fig. 5). We speculated that these highly expressed genes might play important roles in repairing indole-induced protein damage and degradation conditions.

**DISCUSSION**

It has been reported that QS regulators are unstable without their cognate AHL signals, which are essential for...
The proper folding of QS regulators (Zhu & Winans, 2001; Vannini et al., 2002; Zhang et al., 2002; Costa et al., 2012). Recent data also suggested that GroEL/ES might be important for QS in Sinorhizobium meliloti and A. tumefaciens (Chai & Winans, 2009; Marketon & González, 2002). Reduced levels of those chaperone proteins in the GroEL/ES mutant cells led to lower expression of QS-dependent genes, probably because of the improper folding of QS regulators (Chai & Winans, 2009; Marketon & González, 2002). Our previous data demonstrated that indole induced the expression of many chaperone proteins, including GroEL/GroES, and proteases in P. putida (Kim et al., 2013). Chaperone and protease proteins (Lon, GroES and Hsp90) were also induced in A. oleivorans with the addition of indole (Fig. 5), which indicated that such proteins could be involved in the turnover of AqsR in the presence of indole. It is of interest to test the role of those proteins in the degradation and refolding of AqsR. Indole might affect protein folding and stability globally. However, this scenario will be more complex because our data showed that genes that were involved in both chaperone and protease activities were expressed highly at the same time. Thus, some proteins might become more stable and some proteins may disappear rapidly with indole treatment.

Many bacteria have the trp operon, where indole is a precursor for tryptophan biosynthesis (Sasaki-Imamura et al., 2010; Yanofsky, 2007). In P. putida, this trp operon was significantly induced by indole (Kim et al., 2013). Although a high concentration of indole is toxic to cells because it inhibits membrane potentials and lowers ATP production, a low concentration of indole might not be toxic because it could act as a substrate for tryptophan biosynthesis. We also demonstrated that a high concentration of indole altered the NADH/NAD⁺ ratio by changing membrane reduction potentials and the expression of genes that are involved in the TCA cycle in P. putida (Kim et al., 2013). It is worth noting that many chaperone and protease proteins require ATP as an energy source (Cohn et al., 2007; Missiakas et al., 1996), which might affect the folding of some unstable proteins, including AqsR.

Significant amounts of indole are known to be present in the mouse, rat and human gut (up to 1074 μM in the human gut) (Botsford & Demoss, 1972; Sims & Renwick, 1983; Karlin et al., 1985; Zuccato et al., 1993). Many bacteria cells secrete indole (Lee & Lee, 2010). Interestingly, indole is considered a signal molecule in E. coli (Lee et al., 2007; Vega et al., 2012). A recent study proposed that indole signalling alters bacterial subpopulations to antibiotics by activating stress responses, which leads to the E. coli persister formation (Vega et al., 2012; Lee et al., 2010a). SdiA, a solo (orphan) receptor, was proposed to receive the indole signal. However, the following lines of evidence suggested that indole might not be a signalling molecule for SdiA, a QS regulator: (i) the direct binding of indole to SdiA has not been demonstrated; (ii) the indole concentration that is secreted outside cells seems to be too high although many signal molecules work at low concentrations (Lee et al., 2007; Han et al., 2011; Kim et al., 2011; Vega et al., 2012); (iii) indole production in Paenibacillus...
Indole inhibits bacterial QS regulator folding

alvei and E. coli was inhibited by glucose, which was not observed in other QS systems (Vega et al., 2012; Kim et al., 2011); (iv) PpoR, an SdiA homologue in P. putida, could sense the AHL signal (Lee et al., 2010b); (v) a recent report demonstrated that indole changed the gene expression of many genes and biofilm formation in E. coli and Salmonella, where SdiA was not required (Sabag-Daigle et al., 2012); and (vi) high concentrations of indole could not inhibit AHL sensing by SdiA (Sabag-Daigle et al., 2012). Taken together, the actual role of indole as a bacterial signal requires further clarification.

Our previous data also suggested that the AHL–QS regulator complex caused the effect of indole in P. putida (Lee et al., 2010b). Indole enhanced P. putida biofilm formation and inhibited swimming motility, which were not observed when AHL was already bound to the QS regulator (Lee et al., 2010b). This information is consistent with our present data indicating that Agrobacterium TraR that was complexed with AHL could function properly in the presence of indole (Fig. 4). A micromolar concentration of the AHL signal was enough to protect TraR-dependent tral gene expression from a millimolar concentration of indole (Fig. 4). Taken together, QS regulators appear to be stabilized by AHL, but their folding might be affected by indole, which leads to rapid degradation by a Lon-like protease inside cells. When P. aeruginosa and Chromobacterium violaceum were tested to extend this conclusion, QS-dependent production of pyocyanin and violacein (Gallagher et al., 2002; Swem et al., 2009) was inhibited by indole without reducing the QS signal production under indole treatment (data not shown). We speculated that those QS-controlled phenotypic losses and inhibited gene expression by indole were attributable to the interference of QS regulator folding in many bacteria, including A. oleivorans, A. tumefaciens, P. aeruginosa and C. violaceum. Our data led us to conclude that indole influences many bacterial phenotypes by reducing the level of the QS regulator, not by binding to the QS regulator.

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