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Construction of ScbA mutants

Glu to Ala replacement
To mutate Glu to Ala in ScbA, at amino acid positions 78 (DNA position 232–234) and 240 (718–720), the GAG triplet was changed to GCC using the primers E78A and E240A (Table 2 in paper). pIJ6147 was used as template to amplify a 562 bp product with an initial step of 96 °C for 5 min, then 96 °C for 45 s, 65 °C for 35 s and 70 °C for 45 s repeated for 25 cycles, followed by 70 °C for 7 min. This fragment was gel-purified and cloned into pGEM-T Easy vector (Promega), yielding pTE101. Both PCR primers E78A and E240A include one mutation site, Glu78Ala and Glu240Ala respectively, and one AgeI site at the 5’ end of the primer allowed subsequent cloning of the 556 bp DNA fragment from pTE101 into the AgeI site of pIJ6143. pIJ6143 has four AgeI sites; therefore, pIJ6143 was partially digested to obtain a 3.7 kb fragment which deleted the AgeI(II)–AgeI(III) region in scbA (Fig. 3 in paper). pIJ6143 was incubated with 2.5x ethidium bromide for 10 min at 37 °C. One unit of AgeI (New England Biolabs) was added and incubated at 37 °C for 30 min. Calf intestine alkaline phosphatase (CIAP) was added and incubated for 30 min at 37 °C to obtain partially digested DNA fragments. DNA fragments around 3.7 kb were eluted from the gel. The eluted fragments were ligated with a 556 bp AgeI segment from pTE101 and the mixture was used to transform E. coli JM101 (Chung et al., 1989). Plasmids were isolated from the transformants and then digested with BtgI and PvuII to determine the integrity of scbA and the orientation of the insert.

The obtained candidate plasmid, pTE102, has 49 bp deleted between two AgeI sites [from AgeI (I) to (II), Fig. 3 in paper]. The missing 49 bp was replaced by using a modified version of the site-directed ligase-independent PCR-mediated mutagenesis (SLIM) method of Chiu et al. (2004). The primers 49ntF1, 49ntR1, F1 and R1 (see Table 2 in paper) was used for SLIM PCR using pTE102 as template. Primers 49nt F1 and R1 were used to amplify the coding strand, and primers 49ntR1 and F1 were used to amplify the corresponding strand of pTE102 using a mixture of 5 units Taq DNA polymerase (Qiagen) and 0.2 units ProofStart DNA polymerase (Qiagen) with an initial step of 95 °C for 2 min, then 94 °C for 15 s, 53 °C for 25 s and 68 °C for 5 min repeated for 25 cycles, followed by 68 °C for 7 min. PCR products were purified with the Qiagen PCR purification kit and pTE102 was removed by treatment with 10 units DpnI (New England Biolabs). The conditions for SLIM heteroduplex formation were as described by Chiu et al. (2004). An aliquot of 20 µl reaction mixture was used to transform competent E. coli JM101 (Chung et al., 1989). Clones were screened by colony PCR using PCR primer Scebart1 and Scebart2 (Table 2 in paper) to identify a 484 bp PCR product (instead of the 435 bp present in pTE102) with an initial step of 96 °C for 5 min, then 96 °C for 30 s, 58 °C for 30 s and 70 °C for 30 s repeated for 25 cycles, followed by 70 °C for 7 min and the candidate was confirmed by DNA sequencing (MWG) within the scbA gene using scba414 and 628-re-c primers, and named pTE103. The EcoRI 1.28 kb fragment from pTE103 was subcloned into the EcoRI site of the conjugative vector pSET152 to yield pTE104.

To obtain the single mutation at amino acid position 78 (Glu to Ala), primers E78A20 and E78A20anti (Table 2 in paper) were used in SLIM PCR as indicated above to amplify DNA from pIJ6143. Primer E78A20c (Table 2 in paper), which contains the GCC triplet at the 3’ end (instead of GAG present in wild-type scbA), and primer 628-re-c (Table 2) were used to screen for the modified scbA with an initial step of 96 °C for 5 min, then 96 °C for 30 s, 65 °C for 30 s and 70 °C for 30 s repeated for 25 cycles, followed by 70 °C for 7 min. The resulting plasmid, designated pTE105, was checked for the
presence of the correct point mutations and the absence of further mutations in the 945 bp scbA by DNA sequencing (MWG). The EcoRI 1.28 kb fragment from pTE105 was subcloned into the EcoRI site of pSET152 to yield pTE106. The single mutation at amino acid position E240A (Glu to Ala) was constructed by the NdeI–Bbpl DNA fragment from pTE103 and subcloned into pIJ6143, giving pTE107. The EcoRI 1.28 kb fragment from pTE107 was subcloned into the EcoRI site of pSET152, resulting in pTE108.

Arg to Lys replacement
To mutate Arg to Lys in ScbA at amino acid position 243, the CGG triplet was changed TO AAG (DNA position 727–729) using SLIM-PCR as indicated above, except that primers R243K and R243K-anti (Table 2 in paper) were used in the SLIM-PCR to amplify DNA from pIJ6143. Primer R243K-c (Table 2 in paper), which contains the CTT triplet at the 3’ end (instead of CGG present in wild-type scbA), and primer scba414 (Table 2) were used to screen for the modified scbA with an initial step of 96 °C for 5 min, then 96 °C for 30 s, 57 °C for 30 s and 70 °C for 35 s repeated for 25 cycles, followed by 70 °C for 7 min. The resulting plasmid, designated pTE109, was analysed for the presence of the correct point mutations and the absence of further mutations in the 945 bp scbA by DNA sequencing. The EcoRI 1.28 kb fragment from pTE109 was subcloned into the EcoRI site of the conjugative vector pSET152 to yield pTE110.

References