Evaluation of the wild type and Tn5::gusA mutants of *Enterobacter* sp. R29 and *Pseudomonas aeruginosa* AMAAS57 on growth and yield of groundnut

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Abstract: To Evaluate of the wild type and Tn5::gusA mutants of Enterobacter sp. R29 and Pseudomonas aeruginosa AMAAS57 on growth and yield of groundnut. Molecular marker gusA, was introduced into AMAAS57 and Rh 29 and recipients were selected by kanamycin chloramphenicol resistance, respectively. All the mutants were characterized. All the selected 20 gusA mutants isolated were sown with groundnut in pots in triplicates under unsterile soil conditions for studying the nodule occupancy under different salinity induced condition. The study was conducted in Directorate of Groundnut Research, Junagadh in 2010. Molecular marker gusA, was introduced into wild type AMAAS57 and Rh 29. Mutants were characterised and selected for the pot trial with groundnut plant. The plant samples, rhizosphere and rhizoplane soil samples were collected at 45 and 90 DAS for various observations of crop growth and population of gusA tagged mutants. Root and shoot length and biomass; nodule number and dry weight; nodule occupancy by gusA marker; pod yield (g/p), root and shoot dry weight (g/p), etc. were observed. The result showed, with increase in salinity there was decrease in nodule occupancy by R29. However, mutation improved the nodule occupancy at elevated salinity as compared to wild type. Monitoring the population of the wild type and Tn5::gusA mutants of Pseudomonas aeruginosa AMAAS57 and Enterobacter sp. R29 and their mutants in the rhizosphere of groundnut, cultivar GG2 at 0 and 2 EC of salinity revealed that the population of wild type was much lower as compared to mutants without salinity in the rhizosphere at 45 DAS. However, with increase in salinity, the population of the wild type was better. However, there was reduction in the population of mutants. Population of Enterobacter sp. R29 and its mutants were much better in the rhizoplane across salinity and 45 and 90 DAS. There were wide variations in the population of the mutants of Pseudomonas aeruginosa AMAAS57 both in the rhizosphere and rhizpoplane across salinity and maturity of the plant. The variation of the population of the mutants might be due to alternation in the genes responsible for colonization and also in the genes responsible for salinity tolerance in Enterobacter sp. R29 due to insertion of Tn5::gusA..

Keyword: *Molecular marker gusA, Molecular tagging, Tn5::gusA mutants, nodule occupancy, rhizosphere competence.*

1. INTRODUCTION:

Advances in recombinant DNA technology and classic strain engineering approaches have enabled modifications in organisms. Tn5 is a versatile tool that can generate mutants with altered functions. Its transposition in bacterial DNA is random and results in single-site, non-leaky, polar mutations with a selectable phenotype. There are reports where Tn5 mutants have exhibited better activity as compared to NBRI1108 strain. NBRI1108T of *P.*

putida showed enhanced temperature tolerance and toluene-resistance [6]. NBRI1108T of *Azospirillum lipoferum* has been reported to significantly alter indole acetic acid (IAA) production [1], while in another study, Tn5 NBRI1108T expressed over four-fold more bacteroid cytochrome-c oxidase in *Bradyrhizobium japonicum*[10].
β- Glucuronidase is a system that uses the gusA gene coding for the hydrolytic enzyme GUS [7] as a marker for the study of competitive ability of strains of rhizobia [16, 2]. Root nodules occupied by strain marked with gusA gene can be identified by development of a coloured product upon incubation of the root system; crushed nodule extract in an appropriate enzyme substrate.
Assessing GUS is proving to be highly effective marker for ecological studies. There is no background activity in either plant or any rhizobial strain tested. The assay is technicaly simple. A root system is dug up and incubated in a phosphate buffer containing GUS substrate X-glu (5-bromo-4chloro-3-indoyl β-D-glucuronide). GUS cleaves X-glu to release an indoxyl derivative, which, on dimerization, changes to an indigo precipitate. Therefore the presence and physical location of the marked strain can be detected by observing the distribution of the blue colour. Finally, glucuronide substrates do not normally occur either in plants or in bacteria.
Insertational inactivation of the gene (s) by gusA results in polar effect on the downstream gene (s) in a multigene operon [4].the mutants are usually non- leaky and stable because once the plasmid is transferred by mating to a different recipient strain it is no longer able to replicate. Similar observation was also noticed by Wilson *et al*. [16]. Metabolite Profiling Reveals Abiotic Stress Tolerance in Tn5 Mutant of *Pseudomonas putida* and Identification of abiotic stress tolerant metabolites from the NBRI1108T suggest that Tn5 mutagenesis enhanced tolerance towards high temperature and drought. Tolerance to drought was further confirmed in greenhouse experiments with maize as host plant, where NBRI1108T showed relatively high biomass under drought conditions [14]. The analysis of an A. brasilense Tn5 mutant displays a higher tolerance to oxidative stress agents. In contrast to the isogenic parent strain, colonies of the mutant are weakly stained by Congo red added to solid media and are impaired in flocculation [3].

2. MATERIALS AND METHODS

2.1 Molecular tagging:

In order to study the nodule occupancy of *Rhizobium* isolate Rh29 and rhizosphere competence of *P. fluorescens* AMAAS57, *gus*A, was introduced into AMAAS57 and Rh 29 and recipients were selected by kanamycin chloramphenicol resistance, respectively. Biperental mating method of Simon *et al.* [13] was carried out. *E. coli* (A31) carrying Tn5:: *gus*A (Amp^r) and the recipient strains were grown in Tryptone-Yeast extract broth containing 100µg/ml ampicillin and without antibiotics. After 24h of growth, both the broth was centrifuged and pallets were dissolved in 0.5 ml of fresh Tryptone-Yeast extract broth. Bacterial conjugation between donor (*E. coli* A31) and recipient was carried out by keeping a donor: recipient ratio of 1;1. After 10 h of incubation at 28 ±2°C, the growth was scrapped and dissolved in 900 ml Tryptone-Yeast extract broth and diluted upto 10^{-4} and plated.

2.2 Selection of mutants:

E.coli was sensitive to kanamycin and chloramphenicol whereas AMAAS 57 and Rh 29 were resistant to kanamycin and chloramphenicol, respectively. Therefore, AMAAS 57 mutants were selected onto Tryptone-Yeast extract agar plates containing kanamycin (100µg/ml) and ampicillin (100µg/ml) along with IPTG (4µl of 200mg/ml stock) and X-glu (40µl of 20mg/ml stock) and Rh 29 mutants were selected onto Tryptone-Yeast extract agar plates containing chloramphenicol (100µg/ml) and ampicillin (100µg/ml) along with IPTG (4µl of 200mg/ml stock) and X-glu (40µl of 20mg/ml stock). An aliquot of the dilution of 10^{-2}, 10^{-3} and 10^{-4} were plated onto the TY plates and incubated at 28 ±2°C for 48 h. Putative blue colonies were picked up as mutants.
Mutants were grown on Tryptone-Yeast extract agar plates (Amp^{100} Kan^{100} for AMAAS57 mutants and amp^{100} chl^{100} for Rh29 mutants) for 5-6 generations to provide stability and maintained on Tryptone-Yeast extract agar stab containing antibiotics at 4° C in refrigerator.

2.3Stock solution of IPTG and X-gul:

Stock solutions of IPTG (Isopropyl β-D thiogalactoside, Sigma) was prepared at concentration of 200mg/ml in deionized water and sterilized by syringe filter, as described by Maniatis *et al*. [9] and stored at -20°C.
X- gluA (5-bromo-4-chloro-3-indoyl β-D-galactoside, Sigma) was prepared at the concentration of 20mg/ml of Dimethyl formamide as described by Maniatis *et al*. [9] and stored at -20°C.
Antibiotic stock: ampicillin, kanamycin and chloramphenicol stocks were prepared at 50 mg/ml concentration by dissolving in deionized water and sterilized by syringe filter as described by Maniatis *et al*. [9].

Composition of Tryptone-Yeast extract agar:

Tryptone:	10gm/lit
Yeast extract:	5gm/lit
K_2HPO_4:	3gm/lit
Sucrose:	50gm/lit
Agar:	20gm/lit

2.4 Characterization of mutants:

A total of 50 mutants were obtained. All the mutants were characterized for the production of IAA, siderophore and solubilization of tri-calcium phosphates.

2.5 Pot trial under un-sterile soil conditions for studying the nodule occupancy:

In the present study, gusA marker was used to monitor the introduced Rhizobium isolate Rh29 and P. fluorescens AMAAS57, in the root nodules of groundnut crop and to monitor the population in soil at 45 DAS. The mutants were characterized further for the production of siderophore, IAA etc. as described earlier.
All the 20 gusA mutants isolated were sown in pots in triplicates under unsterile soil conditions for studying the nodule occupancy.

The details of the experiments:

1. Groundnut crop: GG2
2. Mutants: Rh 29-9, Rh29-35, Rh 29-45, Rh 29-56, AMAAS57-5, AMAAS57-9, AMAAS57-15 and AMAAS57-20
3. Wild type: Rh 29 and AMAAS 57
4. Replication: 3
5. Salinity dose: 0 and 2 dS/m
6. Duration of sampling: 45 and 90 days after germination

2.6 Maintenance of pots:

One week after germination, enough moisture was maintained in the pots by watering at regular interval. The pots were kept in sunlight where day temperature was 30-35° C.

3. OBSERVATION:

The plant samples, rhizosphere and rhizoplane soil samples were collected at 45 and 90 DAS for various observations of crop growth and population of gusA tagged mutants. Root and shoot length and biomass; nodule number and dry weight; nodule occupancy by gusA marker; pod yield (g/p), root and shoot dry weight (g/p), etc. were recorded.

3.1 Population of introduced organisms:

Rhizosphere:

Rhizosphere soil (the volume of soil adjacent to and influenced by root) sample was collected at 45 and 90 DAS and dilution was made upto 10-5. An aliquot of the dilution of 10-3, 10-4 and 10-5 were plated onto the TY plates containing kanamycin (100µg/ml) and ampicillin (100µg/ml) along with IPTG (4µl of 200mg/ml stock) and X-glu (40µl of 20mg/ml stock) for AMAAS 57 mutants and chloramphenicol (100µg/ml) and ampicillin (100µg/ml) along with IPTG (4µl of 200mg/ml stock) and X-glu (40µl of 20mg/ml stock) for Rh 29 mutants and incubated at 28 ±2oC for 48 h. The blue colonies were counted and expressed as a log number of cells g-1.

Rhizoplane:

Ten gram of the root sample was collected at 45 and 90 DAS and dilution was made upto 10-6. An aliquot of the dilution of 10-4, 10-5 and 10-6 were plated onto the TY plates containing kanamycin (100µg/ml) and ampicillin (100µg/ml) along with IPTG (4µl of 200mg/ml stock) and X-glu (40µl of 20mg/ml stock) for AMAAS 57 mutants and chloramphenicol (100µg/ml) and ampicillin (100µg/ml) along with IPTG (4µl of 200mg/ml stock) and X-glu (40µl of 20mg/ml stock) for Rh 29 mutants and incubated at 28 ±2oC for 48 h. The blue colonies were counted and expressed as a log number of cells g-1.

Statistical analysis

Statistical analysis of the experimental data was done following the SPSS package. All results were subjected to the least significant difference (LSD) test between means. The correlation co-efficient between a pair of means of related traits was determined. Standard deviation was determined following the standard procedures whatever required. The population densities of the isolates were estimated after log transformation of individual estimation.

4. RESULT :

4.1. Evaluation of the wild type and Tn5::gusA mutants of *Enterobacter* sp. R29 and *Pseudomonas aeruginosa* AMAAS57 on growth and yield of groundnut

Wild types and four each of the Tn5::gusA mutants of Enterobacter sp. R29 and Pseudomonas aeruginosa AMAAS57 were evaluated for determining the population dynamics, rhizosphere competence and nodule occupancy and effect on growth and yield in potted conditions at two levels of salinity (0 and 2 EC of irrigation water). The population was also monitored to ascertain when mutation had in impact on the proliferation of the organisms in the rhizosphere. Results (Table 1) indicated that seed bacterisation of groundnut by Pseudomonas aeruginosa AMAAS57 and Enterobacter sp. R29 and their Tn5::gusA mutants affected the growth and yield of groundnut, cultivar GG2. Inoculation of wild type and mutants of Enterobacter sp. R29 resulted in significantly reduced nodule number/plant at 45 DAS when inoculated with mutants like R29-9 and R29-35 without salinity

(Table 1) as compared to uninoculated control but at par with the wild type. Significantly reduced plant biomass was also obtained without salinity when inoculated with the wild type and Tn5::gusA mutants of R29 as compared to uninoculated control but at par among the treatments. Increase in salinity, however, from 0 to 2 EC of irrigation water reduced the shoot length of groundnut when inoculated with Tn5::gusA mutants of Enterobacter sp. R29-45. It was also found that level of yield and other growth parameters were low in all the treatments at 2 EC level of salinity. However, as compared to corresponding controls, there was improvements in growth and yield parameters when inoculated with mutants and wild type of Enterobacter sp. R29 (Table 1). In case of wild type and Tn5::gusA mutants of Pseudomonas aeruginosa AMAAS57, there was significant reduction in the shoot length, pod yield, nodule number and plant biomass when inoculated with mutants like AMAAS57-15 and AMAAS57-20 as compared to uninoculated control and wild type and other mutants without application of saline water (Table 1). But when level of salinity increased from 2 to 2 EC, there was significantly higher pod yield when inoculated with Tn5::gusA mutants of Pseudomonas aeruginosa AMAAS57-20 (Table 1). There were, however, mixed response for wild type and mutants at 2 EC level of salinity.

Table 1. Evaluation of the effect of Tn5::*gusA* mutants of *Enterobacter* sp. R29 and *Pseudomonas aeruginosa* AMAAS57 on the growth and yield of groundnut, cultivar GG2 at different levels of salinity (potted conditions, Summer 2011)

Treatments and salinity	Shoot Length (cm/p)	Root Length (cm/p)	Pod yield (g/p)	Nodule Number/ p	Shoot Dry weight (g/p)	Root dry weight (g/p)	Plant biomass (g/p)
0 EC + control	20.8	14.3	7.83	30.7	8.55	0.54	9.09
0 EC + R29	19.0	14.5	6.00	23.7	6.24	0.25	6.49*
0 EC + R29-9	20.5	15.5	6.50	12.0*	5.21*	0.31	5.52*
0 EC + R29-35	18.8	15.5	5.50	14.2*	6.84	0.31	7.16
0 EC + R29-45	20.0	16.5	7.67	24.3	4.80*	0.41	5.19*
0 EC + R29-56	22.2	15.5	8.17	21.7	4.97*	0.28	5.25*
0 EC + AMAAS57	21.3	14.2	6.17	20.3	6.68	0.40	7.10
0 EC + AMAAS 57-5	20.3	14.0	6.50	17.3*	6.59	0.38	6.95
0 EC + AMAAS 57-9	20.0	16.2	4.67	24.0	6.37	0.50	6.87
0 EC + AMAAS 57-15	12.3*	11.7	4.50*	6.5*	2.86*	0.24	3.10*
0 EC + AMAAS 57-20	13.0*	14.5	3.00*	7.2*	2.96*	0.25	3.21*
2 EC + Control	13.2	14.0	2.00	2.3	1.27	0.22	1.49
2 EC + R29	13.0	15.3	3.00	30.0*	3.89*	0.42	4.30*
2 EC + R29-9	13.0	17.0	7.00*	4.0	4.66*	0.29	4.96*
2 EC + R29-35	14.0	19.0	3.00	12.0	3.21	0.47	3.68
2 EC + R29-45	8.0*	14.0	3.00	2.0	0.62	0.13	0.75
2 EC + R29-56	13.0	15.0	3.00	4.0	1.86	0.21	2.07
2 EC + AMAAS 57	13.8	15.0	6.00*	18.8*	6.53*	0.58	7.10*
2 EC + AMAAS 57-5	14.0	16.0	3.00	4.0	2.40	0.30	2.70
2 EC + AMAAS 57-9	12.7	17.0	6.33*	6.7	3.85*	0.47	4.32*
2 EC + AMAAS 57-15	13.0	18.0	5.00	6.0	5.05*	0.45	5.50*
2 EC + AMAAS 57-20	16.0	24.0	9.00*	1.0	3.58	0.35	3.93

CD (0.05)	3.0	NS	3.25	12.4	2.35	0.18	2.44

a) Nodule occupancy:

Evaluation of the nodule occupancy of Enterobacter sp. R29 by molecular marker like Tn5::gusA in groundnut at 45 DAS and at harvest indicated that at 45 DAS, the nodule occupancy varied from 49% (wild type) -92% (mutants) at without application of salinity as compared to 21% (wild type) – 90% (mutants) when salinity was increased to 2 EC (Table 2). The results indicated that there was significant improvement in the nodule occupancy of mutants as compared to the wild type at 45 DAS. However, the situation changed when nodule occupancy was evaluated at 90 DAS. The nodule occupancy varied from 96% (wild type) to 100% (mutants) at 0 EC and it varied from 85% in wild type to upto 100% in case of mutants (Table 2).

Table 2. Evaluation of the nodule occupancy by theTn5::gusA mutants and wild type of Enterobacter sp. R29

Mutants/ wild type	Nodule Occupancy (%)			
	45 days after germination		90 days after germination	
	0 dSm^{-1}	2 dSm^{-1}	0 dSm^{-1}	2 dSm^{-1}
R29	48.43	20.40	96.30	85.21
R29-9	75.00	69.23	68.75	10.00
R29-35	92.31	60.00	100.00	87.50
R29-45	85.70	90.00	100.00	100.00
R29-56	59.09	53.30	61.54	75.00

b) Bacterial population densities

The population of the rhizobacteria, Pseudomonas aeruginosa AMAAS57 and Enterobacter sp. R29 and their mutants were monitored in the rhizosphere of groundnut, cultivar GG2 at 45 DAS and at harvest at 0 and 2 EC of salinity (Table 3). It was found that the population of wild type and mutants of Enterobacter sp. R29 varied from 2X103 cfu/g (wild type) to upto 1X104 cfu/g (mutants) without salinity in the rhizosphere at 45 DAS (Table 3). Increase in salinity from 0 to 2 EC resulted in better population in wild type (4X104 cfu/g) but reduced with the mutants (upto 4X103 cfu/g). At the time of harvest almost similar trend was noticed for rhizosphere population (Table 3). Population of Enterobacter sp. R29 and its mutants were much better in the rhizoplane and it varied from 1X104 cfu/g -1X106 cfu/g (wild type) to 1X104 cfu/g -3X106 cfu/g (mutants) across salinity and 45 and 90 DAS (Table 3). Monitoring the population of Pseudomonas aeruginosa AMAAS57 revealed that in most of the cases population of mutants could not be detected. However, population varied from 3X102 – 4X103 cfu/g (wild type) to upto 70-7X103 cfu/g (mutants) in the rhizosphere across salinity and maturity of the plants (Table 3). In the rhizoplane, the population of Pseudomonas aeruginosa AMAAS57 and its Tn5::gusA mutants varied from 2X104-1X105 cfu/g (wild type) to upto 1X104-2X106 cfu/g (mutants) across salinity and maturity of the plant (Table 3)

Table 3. Population densities of wild type and Tn5::*gusA* mutants of *Enterobacter* sp. R29 and *Pseudomonas aeruginosa* AMAAS57

Wild type / mutant	Rhizosphere (cfu/g)				Rhizoplane (cfu/g)			
	45 days after germination		90 days after germination		45 days after germination		90 days after germination	
	0 dSm^{-1}	2 dSm^{-1}	0 dSm^{-1}	2 dSm^{-1}	0 dSm^{-1}	2 dSm^{-1}	0 dSm^{-1}	2 dSm^{-1}
R29	2x 10^3	4x10^4	1x 10^3	2x 10^3	1x 10^4	1x 10^6	1x 10^6	ND
R29-9	ND	3x 10^3	4x 10^5	7x 10^3	2x 10^4	3x 10^6	ND	ND
R29-35	1x 10^3	4x 10^3	ND	1x 10^2	1x 10^5	1x 10^5	1x 10^6	1x 10^4
R29-45	1x 10^4	ND	1x 10^3	1x 10^3	3x 10^4	1x 10^6	1x 10^4	ND
R29-56	1x 10^3	ND	1x 10^5	1x 10^2	ND	ND	1x 10^4	2x 10^4
AMAAS57	1x 10^3	ND	4x 10^3	3x 10^2	2x 10^4	ND	1x 10^6	ND
AMAAS57-5	ND	ND	ND	1x 10^2	ND	ND	1x 10^4	2x 10^6

AMAAS57-9	ND	ND	ND	2x 10^2	ND	ND	2x 10^4	ND
AMAAS57-15	ND	ND	4x 10^3	7x 10	1x 10^4	1x 10^4	ND	ND
AMAAS57-20	1x 10^3	7x 10^3	ND	14x10^2	ND	ND	ND	ND

ND= not detected

5. DISCUSSION:

Studying the population dynamics and establishment using molecular markers

Molecular markers are widely used to study the establishment of inoculants strains in the rhizosphere of many crops species [12] as intrinsic antibiotic resistance patters are not that authentic in counting the population. The molecular markers include Tn5::lacZ, Tn5::gusA, Tn5::lucABC, Tn5::gfp, Tn5::rfp, etc. [12].
To monitor the population of the inoculants strains and their rhizosphere competence and nodule occupancy, four each of the isogenic Tn5::gusA mutants of Enterobacter sp. R29 and Pseudomonas aeruginosa AMAAS57 were evaluated at two levels of salinity (0 and 2 EC of irrigation water). Evaluation of the nodule occupancy of Enterobacter sp. R29 by molecular marker like Tn5::gusA in groundnut at 45 DAS and at harvest indicated that at 45 DAS, the nodule occupancy varied from 49% (wild type) - 92% (mutants) at without application of salinity as compared to 21% (wild type) – 90% (mutants) when salinity was increased to 2 EC. This indicated that with increase in salinity there was decrease in nodule occupancy by R29. However, mutation improved the nodule occupancy at elevated salinity of 2EC as compared to wild type as evident from higher level of nodulation in mutants as compared to wild type Enterobacter sp. R29 at 45 DAS. However, evaluation at harvest revealed that there was 85-96% nodulation by wild type as compared to 100% in mutants. This might be due to polar effect on the regulatory elements (repressor) on nod functions due to insertion of Tn5::gusA.
Monitoring the population of the wild type and Tn5::gusA mutants of Pseudomonas aeruginosa AMAAS57 and Enterobacter sp. R29 and their mutants in the rhizosphere of groundnut, cultivar GG2 at 45 DAS and at harvest at 0 and 2 EC of salinity revealed that the population of wild type was much lower as compared to mutants without salinity in the rhizosphere at 45 DAS. However, with increase in salinity from 0 to 2 EC, the population of the wild type was better. However, there was reduction in the population of mutants. Population of Enterobacter sp. R29 and its mutants were much better in the rhizoplane across salinity and 45 and 90 DAS.
There were wide variations in the population of the mutants of Pseudomonas aeruginosa AMAAS57 both in the rhizosphere and rhizpoplane across salinity and maturity of the plant.

6. CONCLUSION:

The variation of the population of the mutants might be due to alternation in the genes responsible for colonization and also in the genes responsible for salinity tolerance in Enterobacter sp. R29 due to insertion of Tn5::gusA.
Transposable genetic elements carrying antibiotic resistance genes have proven to be extremely useful tools in bacterial genetics [8]. The transposition of a Tn element into a gene generally inactivates it and mutations are usually non leaky and stable. Such insertions also exert polar effects on downstream genes of the operons [4].
Vincent et al. [15] reported the use of site directed Tn5 mutagenesis to assess the role of DAPG (2, 4 -diacetylphloroglucinol) produced by P. aureofaciens Q 2-87 to suppress G. graminis var. tritici. They could isolate two mutants of Q 2-87 with altered antifungal activity. One mutant Q 2-87 :: Tn5-1, did not inhibit G. graminis var. tritici in vitro and did not produce DAPG. Complementation analysis with two cosmids, isolated from the genomic library, coordinately restored the antifungal activity and DAPG production in vitro. Subcloning and deletion analysis of these cosmids identified a 4.8 kb genomic region responsible for DAPG production and antifungal activity. Fenton et al. [5] used Tn5 mutagen to derive DAPG deficient mutant (F113 G22) from Pseudomonas sp. strain F113.
Inactivation of repressor could give overproducing strains having enhanced biocontrol ability.

O'Sullivan and O'Gara [11] could inactivate the Fur-like repressor of a fluorescent pseudomonad which has been shown to improve the inhibitory characteristics of the strains under high-iron conditions. Therefore, the improvement in the nodulation in the mutants of Enterobacter sp. R29 might be due to inactivation of repressor proteins regulating the nodulation process.

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