STRUCTURAL MODIFICATION OF *ESCHERICHIA COLI* PEPTIDOGLYCAN INDUCED BY BICYCLOMYCIN

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We have studied the modification of *Escherichia coli* peptidoglycan induced by bicyclomycin. For this purpose liquid chromatography for peptidoglycan analysis has been used. The main alteration found was an increase of diaminopimelyl-diaminopimelyl bridge containing subunits. Our results show that bicyclomycin impairs the normal breakage of that interpeptidic bond, whose cleavage is needed for the normal remodeling of peptidoglycan and cell growth. Based on the analysis of the possible structure of diaminopimelyl-diaminopimelyl bond and bicyclomycin, we propose a hypothesis on the mechanism of action of bicyclomycin.

Bicyclomycin is an antibiotic which was first isolated from *Streptomyces sapporonensis* ATCC 21532, by the Research Laboratories of Fujisawa Pharmaceutical Co., Ltd.[1]. This antibiotic is active against a number of Gram-negative bacteria, but ineffective against Gram-positive microorganisms[2]. TANAKA *et al.*[3] reported that bicyclomycin inhibited protein synthesis and particularly synthesis of envelope proteins, mainly lipoprotein. These authors found that the levels of murein-bound lipoprotein in bicyclomycin-treated *Escherichia coli* cells, were decreased. Morphological studies carried out by SOMEYA *et al.*[4] have shown that bicyclomycin induces the formation of blebs on the cell surface as well as a highly undulated outer membrane. However, later work by HIROTA *et al.*[5] demonstrated that mutants lacking lipoprotein could grow normally under a variety of conditions. These data indicated that, at least, the main killing action of bicyclomycin is not at the lipoprotein level[6]. The existence of seven minority proteins located in the cytoplasmic membrane of *E. coli* that bound ^{14}C-bicyclomycin was later shown[6]. These bicyclomycin-binding proteins (BBPs) were shown to be distinct from penicillin-binding proteins (PBPs) previously described by SPRATT[7]. Nevertheless, despite those findings, the mechanism of action of bicyclomycin remains unknown although some reasonable hypotheses have been made with regard to the mode of action of the antibiotic with its target proteins[8~10]. In this paper we report the structural modifications of *E. coli* peptidoglycan induced by bicyclomycin. From our data, we put forward a hypothesis on the mode of action of the antibiotic.

Materials and Methods

Bacterial Strains and Growth Conditions

E. coli ATCC 27166 (a mutant hypersensitive to bicyclomycin[2]) and *E. coli* W7 (*dapA, lysA*) were used in this study. Both strains were grown in L medium supplemented with 4 g/liter of D-glucose and, only for W7 strain, 5 mg/liter of 2,6-*meso*-diaminopimelic acid. Cells were grown under forced aeration and established conditions (see the legend of Fig. 1 and results) and were collected by rapid cooling and centrifugation.

Fig. 1.   Action of bicyclomycin on the growth of *E. coli* ATCC 27166 (A) and *E. coli* W7 (B).

Upward arrows: addition of bicyclomycin at concentrations (μg/ml) for A: (●) 0, (○) 4, (▲) 8, (■) 12, (□) 20; for B: (●) 0, (○) 25, (▲) 30, (■) 40.   Cells were collected (downward arrows) and treated for peptidoglycan analysis.   In the case of W7 strain, cells had been labeled with [³H]-2,6-*meso*-di-aminopimelic acid 15 minutes before harvesting.
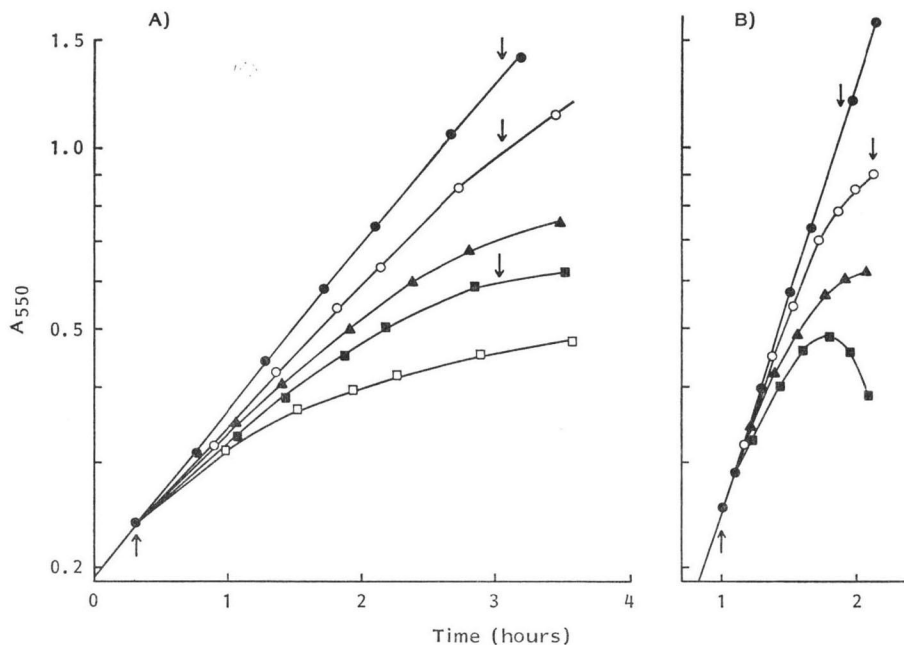
Radioactive Labeling of *E. coli* Peptidoglycan

E. coli W7 cells growing exponentially in a culture were treated with [³H]-2,6-*meso*-diaminopimelic acid (Commisariat à l'Energie Atomique, Gif-sur-Yvette, France) at a final concentration of 5 μCi/ml.   The addition of the radioactive precursor was done as illustrated in Fig. 1.   The incubation was continued for 15 minutes and after that cells were collected as described above.

Isolation and Structural Analysis of Peptidoglycan

Centrifuged cells were treated for the isolation of peptidoglycan as described previously[11].   This procedure includes the treatment of peptidoglycan by pronase which cleaves off most bound lipoprotein polypeptide, leaving only a lysyl-arginine residue attached to peptidoglycan.   Deproteinized peptidoglycan was hydrolyzed by "*Chalaropsis*" muramidase as described[12].   Peptidoglycan subunits were resolved by high pressure liquid chromatography (HPLC).   The procedure for peptidoglycan fractionation by HPLC as well as the structural analysis and identification of chromatogram peaks have been reported previously[13].

Results

Fig. 1A shows the effects of several concentrations of bicyclomycin on *E. coli* ATCC 27166. The antibiotic, at the concentrations used did not produced cell lysis although all concentrations caused a clear inhibition of the growth rate.   Examination of bicyclomycin-treated cells, under light microscopy (not shown) revealed that the antibiotic induced the formation of filaments as described before[2]. Cells were collected as indicated by the downward arrows of Fig. 1A and, subsequently, peptidoglycan was isolated and prepared for HPLC analysis.   The molar fraction of each individual subunit from either bicyclomycin-treated or bicyclomycin-untreated cells is shown in Table 1.   In Table 2, the dif-

Table 1.   Composition of peptidoglycan from *E. coli* ATCC 27166 cells treated with bicyclomycin[a].

Peptidoglycan subunits[b]	Bicyclomycin (μg/ml)		
	0	4	12
1. Tri	10.66	11.66	22.04
2. Tetra (Gly)[c]	1.32	1.46	0.81
3. Tetra	50.67	48.90	36.65
4. Penta (Gly)[c]	ND	ND	ND
5. Dipeptide	3.03	3.31	4.06
6. Penta	1.05	0.80	0.41
7. Tri-Lys-Arg[d]	5.40	4.77	3.79
8. Tetra-tri (Gly)[c]	0.09	0.11	0.41
9. Tri-tri(DAP-DAP)[e]	0.18	0.22	0.81
10. Tetra-tri	1.25	1.39	2.10
11. Tetra-tri(DAP-DAP)[e]	1.32	1.86	3.79
12. Tetra-tetra (Gly)[c]	0.66	0.86	1.89
13. Tri (anh)[f]	ND	ND	ND
14. Tetra-penta (Gly)[c]	0.20	0.20	0.20
15. Tetra-tetra	19.21	18.09	15.01
16. Tetra-penta	0.13	0.33	0.20
17. Tri-tri(DAP-DAP)-Lys-Arg[d,e]	0.07	0.13	0.14
18. Tetra (anh)[f]	0.13	0.27	0.14
19. Tetra-tetra-tri(DAP-DAP)[e]	0.13	0.22	0.72
20. Tetra-tetra-tetra	1.01	0.93	1.40
21. Tetra-tri(DAP-DAP)-Lys-Arg[d,e]	0.92	1.39	2.23
22. Tri-tri(DAP-DAP) (anh)[e,f]	ND	ND	ND
23. Tetra-tri-tri(DAP-DAP)-Lys-Arg[d,e]	0.18	0.18	0.14
24. Tetra-tri (anh)[f]	0.66	0.73	0.20
25. Tetra-tri(DAP-DAP) (anh)[e,f]	0.20	0.27	0.74
26. Tetra-tetra (anh)[f]	0.92	0.99	1.35
27. Tetra-tetra-tri (anh)[f]	ND	0.04	ND
28. Tetra-tetra-tri(DAP-DAP) (anh)[e,f]	ND	0.09	0.14
29. Tetra-tetra-tetra (anh)[f]	0.31	0.35	0.45
30. Tri-tri(DAP-DAP)-Lys-Arg (anh)[d,e,f]	0.33	0.46	0.20

[a]   The table indicates the molar fraction $\times 100$ of each mucopeptide in each condition.

[b]   In this nomenclature the disaccharide part is ommitted for the purpose of clarity.   The prefixes di-, tri-, tetra- and penta- mean disaccharide di-, tri-, tetra- and pentapeptide respectively.

[c]   (Gly) means that the last amino acid of the peptide is glycine substituting to D-alanine.

[d]   Lys-Arg means lysyl-arginine residue which comes from lipoprotein which was bound to the subunit before treating peptidoglycan with pronase.

[e]   (DAP-DAP) means the existence of a diaminopimelyl-diaminopimelyl interpeptide bridge.

[f]   (anh) means anhydro sugar subunit which constitutes the terminal component of a glycan chain.   The ratio non-anhydro/anhydro subunits gives the length of the glycan chain.

ND: Not detected.

ferent subunits of Table 1 have been grouped into families.   From Tables 1 and 2, the following conclusions should be pointed out: i) There was a noticeable increase of disaccharide tripeptide (Table 1, No. 1); ii) the amount of disaccharide tetrapeptide was reduced (Table 1, No. 3); iii) the amount of bis-disaccharide tetrapeptide was also decreased (Table 1, No. 15); iv) disaccharide tripeptide subunits containing lipoprotein were greatly reduced (Table 1, No. 7).   However, v) the total amount of disaccharide peptides, bis-disaccharide peptides and tri-disaccharide peptides, was scarcely affected by bicyclomycin (Table 2, Nos. 1, 2 and 3).   Consequently, vi) the degree of interpeptide cross-linking of peptidoglycan from bicyclomycin-treated cells changed very little (Table 2, No. 4).   This degree of

Table 2. Composition of peptidoglycan from *E. coli* ATCC 27166 cells treated with bicyclomycin.
Data are from Table 1 but here the individual subunits have been grouped into families[a].

Subunit family	Bicyclomycin (μg/ml)		
	0	4	12
1. Monomers[b]	72.3	71.2	68.0
2. Dimers[b]	26.1	27.0	29.2
3. Trimers[b]	1.6	1.8	2.8
4. Bridges[c]	29.4	30.7	35.0
5. DAP-DAP[d]	3.3	4.8	8.9
6. DAP-DAP/Bridges	11.3	15.7	25.5
7. Lipoprotein[e]	6.9	6.9	6.5
8. Lipoprotein D-T[f]	21.7	31.2	41.7
9. Anhydro compounds[g]	2.5	3.2	3.2
10. Glycan chain length[g]	39.2	31.2	31.2

[a]   The table indicates the molar fraction $\times 100$ of each family in each condition.
[b]   Total amount of monomers (disaccaride peptides), dimers (bis-(disaccharide peptides)) and trimers (tri-(disaccharide peptides)).
[c]   Total amount of dimers plus trimers, *i.e.*, those subunits containing interpeptide bridges.
[d]   Total amount of dimers and trimers which have one diaminopimelyl-diaminopimelyl interpeptide bridge.
[e]   Total amount of subunits containing one terminal lysyl-arginine residue derived from hydrolyzed lipoprotein.
[f]   Percentage of cross-linked subunits (dimers and trimers) containing lipoprotein (100%=total subunits containing lipoprotein).
[g]   Anhydro compounds are the terminal subunits of a glycan chain. The ratio non-anhydro/anhydro compounds estimates the chain length, *i.e.*, the number of disaccharide subunits per chain.

cross-linking was not maintained by the usual interpeptide D-alanyl-diaminopimelyl bridges whose amounts actually decreased (see, *e.g.*, Table 1, No. 15); but vii) rather was maintained by the striking increase of cross-linked subunits containing interpeptide diaminopimelyl-diaminopimelyl (DAP-DAP) bonds (Table 2, No. 5 and Table 1, Nos. 9, 11, 17, 19, 21, 25 and 28).   In fact, viii) the ratio between the two kinds of bridges, *i.e.*, DAP-DAP *versus* D-alanyl-diaminopimelyl, increased considerably, the former bridges representing above 25% of the total bridges (Table 2, No. 6).

Fig. 1B, shows the effect of bicyclomycin on the growth of *E. coli* W7 cells.   It can be seen that this strain is not as sensitive to bicyclomycin as is the ATCC 27166 strain.   Indeed, concentrations of bicyclomycin up to 20 μg/ml (not shown) did not produce any noticeable effect on cell growth.   However, concentrations above 25 μg/ml produced retardation of the growth and eventual cell lysis.   Microscopic examination (not shown) revealed that bicyclomycin induced morphological changes in W7 cells.   Such alterations mainly consisted of shortening of the rods to form round or spheroplast-like cells, which exploded with time, but we failed to detect any sort of filaments in bicyclomycin-treated W7 cultures.   However these cells were collected for peptidoglycan analysis when they were still growing at a nearly normal rate.

Table 3 shows the primary structure of peptidoglycan from both bicyclomycin-treated or untreated W7 cells.   Changes in mature peptidoglycan (lanes A and B) are not large, but, there are significant differences between untreated and bicyclomycin-treated cells.   Indeed, the amount of subunits containing interpeptide DAP-DAP bridges decreased upon antibiotic treatment (Table 3, Nos. 5 and 6).   Furthermore, the amount of lipoprotein bound to the peptidoglycan also decreased (Table 3, No. 7), although the percentage of lipoprotein bound to dimers and trimers increased (Table 3, No. 8).   Nevertheless, possibly a finer picture of bicyclomycin-induced alteration on peptidoglycan, emerges

Table 3. Composition of peptidoglycan from *E. coli* W7 cells either untreated (A, C) or treated with 25 μg/ml bicyclomycin (B, D).

A and B represent the molar fraction ×100 of subunit groups from total cell peptidoglycan while C and D show the molar fraction ×100 of subunit groups from *de novo* peptidoglycan labeled with [³H]-2,6-*meso*-diaminopimelic acid.

	Subunit family*	A	B	C	D
1.	Monomers	70.6	71.0	75.7	74.8
2.	Dimers	28.1	28.2	23.8	24.5
3.	Trimers	1.3	0.8	0.4	0.7
4.	Bridges	30.7	29.7	24.7	25.9
5.	DAP-DAP	4.5	3.8	3.8	5.1
6.	DAP-DAP/Bridges	14.7	12.9	15.5	19.6
7.	Lipoprotein	7.4	4.7	5.0	3.1
8.	Lipoprotein D-T	26.1	39.4	38.3	57.6
9.	Anhydro compounds	2.1	1.3	1.2	1.5
10.	Glycan chain length	48.0	74.0	82.3	66.4

* See notes of Table 2.

from pulse-labeling experiments (lanes C and D).   As can be observed, the most noticeable change is that *de novo* peptidoglycan from bicyclomycin-treated cells contained an increased amount of subunits containing the DAP-DAP interpeptide linkage (Table 3, Nos. 5 and 6).   The amount of bound lipoprotein also decreased although, again, the percentage of lipoprotein bound to cross-linked subunits was increased by bicyclomycin treatment (Table 3, Nos. 7 and 8).

Discussion

Cell Alterations Induced by Bicyclomycin

It had been reported previously[2] that bicyclomycin produced mixed effects on the morphology of *E. coli* cells, *i.e.*, the antibiotic produced both round-shaped or spheroplast-like cells and filaments. In this study we have chosen two *E. coli* strains which responded differently to bicyclomycin.   Under our growth conditions the antibiotic exclusively produced filaments in the ATCC 27166 strain, while exclusively round-shaped cells were seen in the W7 strain.   These distinct effects suggest, at first glance, that bicyclomycin might act at different levels, and some of them could predominate in one and others in another strain.   Concomitantly, the effects produced by bicyclomycin on the peptidoglycan structure from either ATCC 27166 or W7 cells could differ from each other.   The finding that bicyclomycin specifically binds to a few proteins of the cytoplasmic membrane of *E. coli*[6] prompted us to initiate this study especially since new technology for peptidoglycan analysis has been developed[13].   Our results ndicate that bicyclomycin induces two main kinds of alterations in peptidoglycan.   First, the antibiotic increases the amount of DAP-DAP bridges in peptidoglycan.   As demonstrated by GLAUNER and SCHWARZ[13], DAP-DAP bridges are very rapidly produced by nascent peptidoglycan.   Also lipoprotein very rapidly gets bound to, and only to, DAP-DAP containing subunits.   Later, the amount of DAP-DAP containing subunits, either with or without lipoprotein decreases, while there is an increase of disaccharide tripeptide containing lipoprotein, obviously because the DAP-DAP linkage is hydrolyzed.   From our data, it seems that bicyclomycin impairs to a great extent the hydrolysis of DAP-DAP bonds.   As a result, the amount of DAP-DAP containing subunits increases.   Furthermore, our data show that the level of lipoprotein bound to cross-linked subunits is higher in antibiotic-treated cells while the amounts of disaccharide tripeptide carrying lipoprotein are greatly reduced, the later could not be generated from lipoprotein bound cross-linked subunits.

The second effect, induced by bicyclomycin on cell peptidoglycan, is of more diffuse characteristics. The amount of cross-linked species containing the more usual D-alanyl-diaminopimelyl interpeptide bridges decreases.   On the other hand, the amount of disaccharide-tripeptide is also increased upon

Fig. 2. A) Structure of bicyclomycin assessed by X-ray crystallography analysis (refs 18 and 19).
B) Proposed structure of the DAP-DAP linkage.
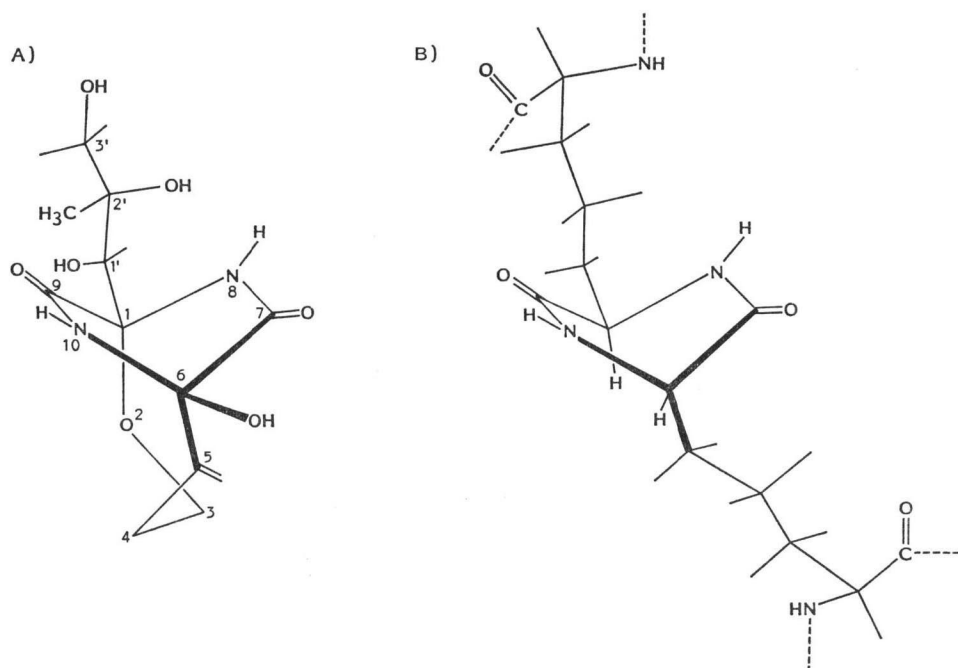
bicyclomycin treatment.   These effects could be due to some difficulty in obtaining the usual cross-linking from *de novo* pentapeptide.   Non utilized pentapeptide excess could undergo degradation to tetra and tripeptide by the action of DD and LD carboxypeptidases.

The DAP-DAP Bond

The existence of di- and trimeric subunits, whose interpeptide bridge is a DAP-DAP bond, was discovered by GLAUNER and SCHWARZ[13].   This is a peptidic (amide) bond as it is broken by acid hydrolysis.   In normal *E. coli* cells, DAP-DAP bonds are responsible for around $12 \sim 17\%$ of the total interpeptide cross-linking of peptidoglycan.   The relative amount of DAP-DAP bonds increases up to 25% in the stationary phase of cell growth[14,15].   However, many questions concerning the structure of this bond and the source of energy for its formation, remain to be answered.   Certainly, this bond involves the two D centers of *meso*-diaminopimelic residues because of the existence of the tetra-tri subunit containing both a DAP-DAP bond and lipoprotein which is linked to the L center of the diaminopimelic residue[16].   The formation of the first amide bond involving one –COOH and one –NH$_2$ groups brings about the formation of a second amide bond which is energetically favored. The final result could be a six member-ring lactam (diketopiperazine ring, see Fig. 2A).

The Mechanism of Action of Bicyclomycin.   An Hypothesis

The targets of bicyclomycin in the bacterial membrane (BBPs) have not yet been characterized. Therefore, nothing is known about the structure of the antibiotic-enzyme complex.   In order to ascertain the place of binding of bicyclomycin in the target enzyme, and the transformations undergone by the antibiotic molecule, studies on the reactivity of bicyclomycin for nucleophilic reagents have been carried out.   In a first study, SOMEYA *et al.*[8] inferred that the active center of bicyclomycin could be the terminal olefinic group that can undergo a nucleophilic attack by thiols forming an adduct whose structure was determined.   These authors concluded that the target protein could be a sulfhydryl-enzyme that might exert a similar nucleophilic attack on bicyclomycin.   Later, MÜLLER *et al.*[17] synthesized a large number of bicyclomycin derivatives.   Some compounds (*e.g.* No. 57 of ref 15) had the terminal olefinic group substituted by an oxime and still retained their biological activity.   Reaction of

thiols with the oxime group would be less favored.   More recent studies by WILLIAMS and his co-workers showed a lack of correlation between simple thiolate susceptibility and biological activity of bicyclomycin.   This indicates that this interesting reaction alone cannot be used as the biomechanistic template[9].   These authors in another article[10] proposed a very interesting mechanism of action of bicyclomycin.   The receptor would be a protease-like enzyme which performs a nucleophilic attack on the ketone 9-carbon of the diketopiperazine ring of the antibiotic.   Thus, the 9, 10-amide bond would be cleaved and an acyl-enzyme intermediate formed.   We propose that the mechanism of action of bicyclomycin is mainly due to the analogy of the diketopiperazine ring of this antibiotic with the lactam involved in the DAP-DAP bonds (see Fig. 2).   The four member bridge between positions 1 and 6 in the molecule of bicyclomycin would maintain the diketopiperazine ring in a forced boat conformation, which is a structural analog of the DAP-DAP bond.   Thus bicyclomycin would inactivate the amidase enzyme responsible for the breakage of the DAP-DAP bond.   As a result DAP-DAP cross-linked subunits would increase in cell peptidoglycan, as shown by our biochemical data.   The excess of DAP-DAP linkage and the difficulty of their breaking would impede, on the one hand, the normal growth of the cell, and, on the other hand, these bonds could impair the formation of the more usual diaminopimelyl-D-alanine interpeptide bonds from nascent pentapeptide, as reflected in our biochemical data. Such impairment could be derived from steric factors that would impede the remodeling of growing peptidoglycan whose structure would remain blocked by uncleavable DAP-DAP bonds.

According to this hypothesis, the lack of sensitivity of Gram-positive bacteria to bicyclomycin could be explained by the fact that most of these bacteria lack γ-*meso*-diaminopimelic acid which impairs the formation of DAP-DAP bond and, in addition, Gram-positive bacteria do not have lipoprotein or outer membrane.

Acknowledgments

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