Systematic evasion of the restriction-modification barrier in bacteria

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Bacteria that are recalcitrant to genetic manipulation using modern in vitro techniques are termed genetically intractable. Genetic intractability is a fundamental barrier to progress that hinders basic, synthetic, and translational microbiology research and development beyond a few model organisms. The most common underlying causes of genetic intractability are restriction-modification (RM) systems, ubiquitous defense mechanisms against xenogenic DNA that hinder the use of genetic approaches in the vast majority of bacteria and exhibit strain-level variation. Here, we describe a systematic approach to overcome RM systems. Our approach was inspired by a simple hypothesis: if a synthetic piece of DNA lacks the highly specific target recognition motifs for a host's RM systems, then it is invisible to these systems and will not be degraded during artificial transformation. Accordingly, in this process, we determine the genome and methylome of an individual bacterial strain and use this information to define the bacterium's RM target motifs. We then synonymously eliminate RM targets from the nucleotide sequence of a genetic tool in silico, synthesize an RM-silent "SyngenicDNA" tool, and propagate the tool as minicircle plasmids, termed SyMPL (SyngenicDNA Minicircle Plasmid) tools, before transformation. In a proof-of-principle of our approach, we demonstrate a profound improvement (five orders of magnitude) in the transformation of a clinically relevant USA300 strain of Staphylococcus aureus. This stealth-by-engineering SyngenicDNA approach is effective, flexible, and we expect in future applications could enable microbial genetics free of the restraints of restriction-modification barriers.

Significance

Genetic engineering is a powerful approach for discovering fundamental aspects of bacterial physiology, metabolism, and pathogenesis as well as for harnessing the capabilities of bacteria for human use. However, the full power of genetic engineering can only be applied to a few model organisms. Biological diversity and strain-level variation in restriction-modification systems are critical barriers keeping most bacteria beyond the full potential of genetics. We have designed a systematic approach to effectively evade restriction-modification systems and successfully applied this approach to a clinically relevant USA300 strain of the human pathogen Staphylococcus aureus. Our results demonstrate the simplicity and effectiveness of this stealth-by-engineering approach, which could enable microbial genetic system design not restrained by innate restriction-modification defense mechanisms.

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Conflict of interest statement: C.D.J. discloses that he has filed and is inventor on pending patent applications (US20190001383 and US20180353244) entitled "Composition of and method for evading bacterial defense mechanisms" and "Production of differentially methylated DNA in E. coli," respectively, relating to the SyngenicDNA and SyMPL methodologies developed and applied in this paper.

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Data deposition: Genomemethylome sequence data for Escherichia coli MC_Forsyth and Staphylococcus aureus USA300 J23100_J23100_Forsyth have been deposited in the REBASE database, http://rebase.neb.com/rebase/rebase.html (under strain nos. J23141 and J23142, respectively).

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bacterial species (5). This may even be an overestimation, as in several cases only a single report documents transformation, and molecular evidence of natural transformation is lacking (5). For all remaining cultivated bacterial species that are of interest, microbiologists must instead develop "artificial" transformation and phenotypic assays to distinguish self- from nonself-DNA via two enzymatic activities: a restriction endonuclease (REase) and a modification methyltransferase (MTase). The REase recognizes the methylation status of DNA at a highly specific DNA target sequence and degrades unmethylated or inappropriate methylated (i.e., non-self) DNA. Its cognate MTase protects the same target sequence across the host’s genome via addition of a methyl group, marking each site as self. RM systems originally evolved as defense mechanisms against invading xenogeneic DNA (8), which is primarily encountered during bacteriophage infection. Consequently, most, if not all, of the currently available approaches to overcome them during genetic engineering are inspired by bacteriophage anti-restriction mechanisms (9, 10). Bacteriophage mechanisms that involve methyl-modification of the phage genome to subvert the host’s RM activities have also been translated into in vitro engineering approaches (9, 10). These can all be referred to as mimicry-by-methylation, as they essentially seek to modify the methylation pattern of a genetic tool to match the desired host and achieve molecular mimicry. There are two common mimicry-by-methylation approaches. (i) Methylate target sites on tools by using in vitro methylation with recombinant MTase enzymes (10), which are currently commercially available for only 37 of >450 known targets (11). (ii) Alternatively, achieve in vivo methylation by passing a plasmid through a related strain that is either restriction enzyme-deficient (10) or a surrogate strain that has been extensively engineered to generate the absence of a recognition motif protected by the MTase of the host, i.e., the plasmid artificial modification (PAM) technique (12). Although these are very effective in some cases (13), owing to the labor-intensive and rigid nature of their underlying design they are often not readily adaptable to other strains due to RM system diversity (SI Appendix, Text S1) and, accordingly, are unsuitable for rapid application to a wide diversity of bacteria (10).

We therefore sought to design a versatile strategy to overcome RM barriers, one suitable for use in a broad range of bacterial species. The problem to be overcome is that in any given bacterial genus, genotyping the sequence of the promoter of RM systems reveals the target sequences recognized are hypervariable and highly species-specific, often even strain-specific (14). RM systems are also extremely diverse and can be differentiated into four types (type I, II, III, and IV), based on their recognized target and, also, subunit composition, cleavage position, cofactor requirements, and substrate specificity (15). Additionally, RM target motifs themselves vary greatly in sequence and length, ranging from 4 to 18 base pairs (bp), with >450 different motifs identified to date (11). It is clear, therefore, that a broadly applicable strategy to overcome RM barriers to genetic engineering will need to be adept at adjusting for RM system variation across different bacterial strains.

Importantly, all REase enzymes demonstrate exquisite specificity in target sequence recognition. This specificity is crucial, as REases are toxic to their hosts’ genome in the absence of their cognate MTases and, consequently, seldom deviate from their recognition sequence (15). In the context of bacterial genetic engineering, this is a critical weakness underpinning the effectiveness of all RM systems. To rapidly exploit the inherent weaknesses of high target specificity, we designed a stealth-based strategy to evade RM system activities entirely. Our approach was inspired by a simple hypothesis: if a piece of DNA lacks the highly specific target recognition motifs for a host’s RM systems, then it is invisible to these systems and will not be degraded upon transformation. As RM defenses recognize genetic tools as xenogeneic DNA by virtue of the methylation status of highly specific target motifs (8), the systematic identification and elimination of such target motifs from the nucleotide sequence of a genetic tool should therefore facilitate the engineering of an in silico syngenic DNA version of the genetic tool’s target DNA sequence and transformation. To succinctly encapsulate our approach, we coined the term “SyngenicDNA” (SI Appendix, Text S2).

One example of the tremendous effort, resources, and time it takes to expand genetic tractability is Staphylococcus aureus, a pathogen with significant relevance to public health, which accounts for over 10,000 deaths per year in the United States (16). Numerous papers describe mimicry-by-methylation approaches that seek to expand tractability to more clinically relevant strains, (e.g., refs. 17 and 18). Here, based on its public health importance, we selected S. aureus (MRSA) USA300 LAC strain (19) to demonstrate proof-of-principle for our stealth-based approaches. We expect these approaches will be adopted by the broader microbiological community, enabling genetic system design no longer restrained by microbial restriction-modification defense mechanisms.

Results

Systematic Generation of SyngenicDNA-Based Genetic Tools. There are four basic steps to produce SyngenicDNA-based genetic tools (Fig. 1): (i) target identification, (ii) in silico sequence adaptation, and (iii) DNA synthesis and assembly. Target identification requires the delineation of each methylated site, with single-base resolution, across an entire bacterial genome (i.e., the methylome) and starts with single-molecule real-time (SMRT) genome and methylome sequencing (SMRTseq) (14). Using methylome data, we delineate each of the recognition motifs protected by the MTases of the host’s RM systems and infer the targets recognized and degraded by their cognate REases (SI Appendix, Text S3). This yields a concise list of a host microbes’ RM targets to be eliminated from the genome through artificial modification (PAM) technique (12). Although these are very effective in some cases (13), owing to the labor-intensive and rigid nature of their underlying design they are often not readily adaptable to other strains due to RM system diversity (SI Appendix, Text S1). Accordingly, are unsuitable for rapid application to a wide diversity of bacteria (10).

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after transformation into the host strain. We have developed two solutions to this potential issue. One solution is to generate a SyngenicDNA E. coli plasmid backbone for each specific microbial host strain (Fig. 1B). However, in routine applications this will increase costs of SyngenicDNA synthesis, and, moreover, the E. coli replicon itself becomes redundant after propagation in E. coli, as it is typically nonfunctional in other bacterial species after transformation. Our alternative solution, therefore, is to remove the E. coli replicon entirely, using minicircle DNA technology, rather than recode it. This approach also increases flexibility because the same E. coli replicon can be used to generate tools for multiple different microbial strains.

**SyngenicDNA Minicircle Plasmid Tools.** Minicircles (MCs) are minimalistic circular expression cassettes devoid of a plasmid backbone (20), which are primarily used in gene therapy applications to drive stable expression of transgenes in eukaryotic hosts. MCs are produced by attaching a parental plasmid (PP) to a transgene cassette, cultivating this construct in an E. coli host grown to high-cell density, inducing construct recombination to form an isolated transgene MC and a separate, automatically degraded, PP containing the E. coli replicon. MCs are then isolated by standard plasmid methods (20). Because any DNA sequence can take the place of the transgene, we hypothesized that MC technology could be repurposed to carry entire microbial plasmids and facilitate the removal of superfluous E. coli replicons from shuttle vectors. We demonstrated that the incorporation of SyngenicDNA sequences into a PP allowed us to create SyngenicDNA Minicircle Plasmid (SyMPL, pronounced “simple”) tools (SI Appendix, Fig. S1). SyMPL tools include replication, selection, and functional domains for operation in a specific non-E. coli host, but lack an E. coli replicon despite being isolated at high concentrations from the MC-producing E. coli strain. In our SyMPL strategy, we attach a synthesized (and assembled) SyngenicDNA tool to the nonSyngenicDNA E. coli PP and propagate this construct in an MC-producing E. coli strain. The induction of MCs via recombination, with concurrent induction of a specific endonuclease that eliminates the PP, allows for easy isolation of a minimalistic SyngenicDNA-based genetic tool ready to transform into the desired host strain (SI Appendix, Fig. SIC).

The majority of laboratory E. coli strains, including the MC-producing E. coli host used in this study, contain three active MTases (Dam, Dcm, and HsdM) that introduce methylation modifications to specific target sites on the host genome (SI Appendix, Fig. S2). The Dam MTase modifies the adenine residue \(^{(\text{m}5\text{A})}\) within the sequence GATC, the Dcm MTase modifies the internal cytosine residue \(^{(\text{m}5\text{C})}\) of the sequence CCWGG (where W is A or T), and the HsdM MTase modifies the internal adenine residue \(^{(\text{m}6\text{A})}\) of the sequence AAGCN\(_2\)GTGC. Therefore, plasmid tools propagated within such E. coli strains, including the minicircle (MC) producing strain (ZYC10P32T2), are modified at these target sequences.

The presence of methylated sites on SyngenicDNA-based tools could activate type IV RM systems upon artificial transformation. Generally, unintentional activation of methyl-targeting type IV systems is avoided by the propagation of plasmids within methyl-deficient E. coli strains such as JM110 (dam\(^{-}\), dcm\(^{-}\), hsdRMS\(^{-}\)) or ER2796 (dam\(^{-}\), dcm\(^{-}\), hsdRMS\(^{-}\)), thus preventing recognition and degradation via these systems. However, such methyl-free E. coli strains are unable to produce MCs since construction of the E. coli MC-producing strain (20) required complex engineering to stably express a set of inducible minicircle-assembly enzymes (the φC31-integrate and the I-SceI homing-endonuclease for induction of MC formation and degradation of the parental plasmid replicon, respectively).

Accordingly, when we repurposed MC technology for bacterial applications, it was also necessary to engineer E. coli MC-producer strains that generate various forms of methylation-free MCs (SI Appendix, Figs. S3–S5). Although a completely methylation-free MC producer could be required when working against type IV systems targeting both adenine- and cytosine-methylated DNA, bacterial RM systems exist with targets that specifically match the E. coli Dam MTase motif (GATC), such as Pin25611FII in *Prevotella intermedia* (14). These systems digest unmethylated Dam sites on genetic tools propagated within methyl-free strains; hence, Dam methylation is protective in these cases. Therefore, we created a suite of E. coli strains capable of producing distinct types of methyl-free MC DNA to account for the inherent variation of RM systems in bacteria and...
maximize the applicability of our SyMPL approach. We applied iterative CRISPR-Cas9 genome editing to sequentially delete Mtase genes from the original E. coli MC-producer strain (dam+, dcm+, hsdM+) (SI Appendix, Fig. S4). These strains produce methylcytosine-free MC DNA (E. coli JM1C; dam+, dcm+, hsdM+), methylcytosine- and methyladenine-free MC DNA except for Dam methylation (E. coli JM2C; dam+, dcm+, hsdM+), and completely methyl-free MC DNA (E. coli JM3C; dam+, dcm+, hsdM+). Depending upon the type IV RM systems identified within a desired bacterial host, one of these strains can be selected and utilized for production of SyMPL tools.

Application of SyngenicDNA and SyMPL Approaches to a Bacterial Pathogen. RM systems are a critical barrier to genetic engineering in most strains of Staphylococcus aureus (21). Based on its public health importance, we selected S. aureus JE2, a derivative of the epidemic methicillin-resistant S. aureus (MRSA) USA300 LAC (19), to demonstrate the efficacy of our stealth-by-engineering approaches. First, we determine the methylome of JE2 using SMRT sequencing and identified this strain’s RM targets. SMRTseq and REBASE analysis of JE2 confirmed the presence of two type-I RM systems recognizing the bipartite target sequences AΩGNhGT and CAΑYNhGT (the modified base within each motif is underlined, and n = any base) (SI Appendix, Table S1) and a type II restriction system (Fig. 2B) featuring a single target motif shown to be methylated within the sequence SCNGS (where S = C or G) (21).

We then applied our SyngenicDNA approach to the E. coli–S. aureus shuttle vector pEPSA5 (Fig. 2A and B). The pEPSA5 plasmid (SI Appendix, Text S4 and Fig. S1) contains a 2.5-kb E. coli replicon (ampicillin-resistance gene with a p15a origin for autonomous replication) and a 4.3-kb S. aureus replicon (chloramphenicol-resistance gene, pC194-derived origin, and a xylose repressor protein gene, xylR) (SI Appendix, Fig. S6). The S. aureus replicon is nonfunctional when pEPSA5 is maintained and propagated within E. coli, and vice versa. Therefore, we modified S. aureus, we modified S. aureus, we modified S. aureus, we modified S. aureus, we modified S. aureus, we modified S. aureus, we modified S. aureus, we modified S. aureus, we modified S. aureus, we modified S. aureus, we modified S. aureus. We synthesized, assembled, and propagated pEPSA5SynJE2 (Fig. 2C), a variant of pEPSA5 that differed by only six nucleotides (99.91% identical at nucleotide level), eliminating three RM target motifs present in the original sequence. We demonstrated a ∼70,000-fold (P = 7.76 × 10−306) increase in transformation efficiency (cfu/µg DNA), using the entirely RM-silent pEPSA5SynJE2Dcm- (propagated in dam−, dcm−, E. coli) compared with the original pEPSA5 plasmid (propagated in dam+, dcm+, E. coli) (Fig. 2D and SI Appendix, Text S5).

Subsequently, we sought to determine whether a further increase in transformation efficiency could be achieved using the SyMPL (minicircle) approach. We used the dam− strains E. coli ER2796 and E. coli JM1C to carry out the minicircle (MC) experiments independently of the type IV system in S. aureus JE2. We generated a SyngenicDNA pEPSA5 minicircle for JE2 (pEPSA5SynJE2MC); 38% smaller than pEPSA5 and free of the original E. coli replicon (Fig. 3A and SI Appendix, Fig. S7).

Most of the S. aureus JE2 RM system targets present on pEPSA5 are in the E. coli replicon (type I: n = 2, and type IV: n = 8) with only a single type I target in the S. aureus replicon (SI Appendix, Fig. S6A); thus the MC approach eliminates two of three type I targets. We investigated (i) whether the SyMPL approach achieves equal or perhaps even greater efficiency than the SyngenicDNA approach and (ii) whether removal of all type I targets is required to achieve appreciable gains in transformation efficiency (compared with a partially SyngenicDNA plasmid that has a single type I target remaining). The original plasmid pEPSA5 (Ddam+) was included in experiments only as a control to systematically understand efficiencies and was not considered a primary comparison. The pEPSA5SynJE2MC variant achieved ∼2 × 107 transformants per microgram DNA, a further 3.5-fold increase (P = 1.78 × 10−8) in transformation efficiency over pEPSA5SynJE2 and a >100,000-fold increase (P = 1.97 × 10−286) compared with the original unmodified pEPSA5 plasmid (propagated in dcm+ E. coli) (Fig. 3B and SI Appendix, Tables S2 and S3).

In SyMPL experiments, by reducing the overall size of MC plasmids, we also increased the number of S. aureus repicons present within the minicircles of DNA used for transformations compared with the micrograms used for full-length plasmids. Increasing the yield of functional repicons per microgram of DNA might be an additional advantage of the MC approach. Thus, to more accurately compare transformation efficiencies between MCs and full-length plasmids, we performed a secondary analysis to adjust the transformation efficiencies from cfu/µg DNA to cfu/ppmol DNA (Fig. 3C and SI Appendix, Table S4). On a cfu/ppmol DNA basis, the MC variant pEPSA5MCdcm− achieved a 436-fold increase in transformation efficiency over the original pEPSA5dcm− (P ≤ 1.0 × 10−306). The increase could be due to the elimination of the two type I motifs along with the E. coli replicon in the MC variant (SI Appendix, Fig. S7), or the smaller MCs passing more readily through reversible pores formed in the S. aureus cell envelope during electroporation, or a combination of both. The relatively small 2.3-fold (P = 1.29 × 10−14) increase in transformation efficiency achieved by MC variant pEPSA5SynJE2MC over the plasmid pEPSA5SynJE2, both of which are completely RM-silent in JE2, favors the first possibility. In contrast, pEPSA5MC and pEPSA5SynJE2MC differed only by the presence or absence of a single type I target, resulting in a 7.76 × 10−306-fold increase in transformation efficiency. Importantly, this suggests that in future applications of the SyngenicDNA approach, if a single target exists in an unadaptable region of DNA, such as an origin of replication or a promoter, its inclusion on an otherwise RM-silent plasmid might have minimal impact on the overall transformation efficiency.

Discussion

We report the development of an approach to circumvent the most common cause of genetic intractability, RM barriers, during microbial genetic engineering. In contrast to current mimicry-by-methylation approaches, ours involves stealth-by-engineering (SI Appendix, Fig. S8). We identify the precise targets of the RM systems within a poorly tractable (or intractable) bacterial strain, eliminate these targets from the DNA sequence template of a genetic tool in silico, via single-nucleotide polymorphisms (SNPs) or synonymous nucleotide modifications, and synthesize a tailor-made version of the tool that is RM-silent with respect to that specific host. This stealth-based SyngenicDNA approach allows for simple reworking of currently available genetic tools and DNA parts to permit them to efficiently operate in bacteria with active RM defenses. Additionally, we have repurposed minicircle technology to generate SyngenicDNA Minicircle Plasmid (SyMPL) tools, which are free from components required for propagation in E. coli but superfluous in the target host. Using a clinically relevant USA300 strain of S. aureus, we have demonstrated the profound improvement in transformation efficiency that can be achieved by systematic evasion of RM systems using these SyngenicDNA and SyMPL approaches.

In future applications, we expect that SyngenicDNA will be most readily applied to genetic tools that are functional in tractable strains, to modify them for use in related strains that are currently intractable or poorly tractable due to RM barriers, e.g., a newly emerging epidemic strain (22) or a newly recognized strain with biotechnological potential. In addition, SyngenicDNA could also facilitate synthetic biology approaches aimed at modular design/assembly of new genetic tools for intractable species where no genetically accessible strain is available (14). Synthetic biology focuses on the construction of biological parts that can be understood, designed, and tuned to meet specific criteria, with the underlying principle that genetic tools should be minimalistic, constructed of modularized parts, and sequence-optimized to allow for compatibility. Standardized formats for genetic tool assembly already exist to facilitate the simple implementation of synthetic tools and distribution of physical
parts between different laboratories (23). However, owing to RM systems variation between different strains of the same bacterial species (14), the design of reusable DNA parts that require physical reassembly for different bacteria is generally not applicable for intractable or poorly tractable strains with active RM systems. SyngenicDNA and SyMPL approaches should change that.

We adopted the core principles of synthetic biology, modularity and compatibility, but also accounted for variation in bacterial RM systems between strains by removing the need for physical assembly of reused parts propagated in other bacterial species. Because SyngenicDNA-based genetic tools require DNA synthesis de novo in the later step, the in silico tool assembly step could be utilized to augment plasmid backbones with additional useful parts (e.g., antibiotic-resistance cassettes, promoters, repressors, terminators, and functional domains, such as transposons or fluorescent markers) or create new tools. Additionally, because there is no requirement for a laboratory to...
physically obtain template DNA for PCR amplification of these additional parts, researchers would only need access to the publicly available DNA sequences of new parts to integrate them into a SyngenicDNA-based genetic tool, which could then be synthesized de novo in context. Notably, compatible replication origins and accessory elements for many cultivable bacterial phyla can be obtained from (i) the NCBI Plasmid Genome database, containing >50,000 complete DNA sequences of bacterial plasmids and associated genes (24), or (ii) the ACLAME database (25) (A Classification of Mobile genetic Elements), which maintains an extensive collection of mobile genetic elements including microbial plasmids from various sources.

In addition to impeding the biotechnological and commercial development of “probiotic” bacterial species (26) and the use of bacteria within industrial biofuel production or industrial processes (27), the limited genetic tractability of many major disease-causing bacteria of relevance to clinical and public health obstructs research in multiple fields. Our SyngenicDNA and SyMPL methods are effective, flexible, and we expect can now be applied to a wide range of bacteria to circumvent innate RM barriers, the most common underlying cause of genetic intractability (SI Appendix, Text S6). Finally, the fundamental methodology developed here will also likely be useful for eva-

Materials and Methods

Microbial Strains and Reagents. E. coli NEBalpha competent cells were purchased from New England Biolabs (NEB). E. coli ER2796 was provided by the laboratory of Rich Roberts (NEB). E. coli MC (ZYCY10PS2T; original minicircle-producing strain) was purchased from System Biosciences (SBI). A full list of reagents is provided in SI Appendix.

Bioinformatics and SyngenicDNA Adaptation in Silico. DNA sequence analysis and manipulations were performed using the Seqbuilder program of the DNASTAR software package (DNASTAR). Details of the bioinformatic tools used for adaptation are provided in SI Appendix.

DNA Synthesis and Assembly of SyngenicDNA Plasmids. A SyngenicDNA variant of the pEPSAS plasmid (pEPSASSyn) was assembled by replacing a 3.05-kb fragment of the original plasmid, encompassing three JE2 RM target sites, with a de novo synthesized DNA fragment that was RM-silent with respect to S. aureus JE2 (Fig. 2 and SI Appendix, Fig. S6). Details of assembly protocols are provided in SI Appendix.

Genome Editing of E. coli MC-Producer Strain. A CRISPR-Cas9/-Red multigene editing strategy was used for scarless MTase gene deletions in E. coli MC (ZYCY10PS2T). Details on construction of a modified anhydrotetracycline-inducible CRISPR-Cas9/-Red system and subsequent genome editing of the E. coli MC strain are provided in SI Appendix.

Production of SyMPL Tools. The 4.3-kb S. aureus replicons of both pEPSAS and the pEPSASSynJE2 were PCR-amplified and spliced to the MC parental plasmid (pMC, Systems Biosciences) to form pEPSASP and pEPSASSynJE2P. Primers and full details are provided in SI Appendix.

S. aureus Transformations. Full details of competent cell preparations and electroporation protocols are provided in SI Appendix.

Statistical Analysis and Data Availability. Statistical analyses were carried out using GraphPad Prism (version 7.04; GraphPad Software) and Stata version 12.1 (StataCorp. 2011. Stata Statistical Software: Release 12. College Station, TX: StataCorp LP). Means with SE (SEM) are presented in each graph. Full details on statistical analyses and data availability are provided in SI Appendix.

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